STUDIES ON THE REVERSIBLE OXIDATION AND PROTEOLYSIS OF HIGHLY-PURIFIED *AZOTOBACTER VINELANDII* POLYNUCLEOTIDE PHOSPHORYLASE

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DEDICATION

To my dearest sweet darling, Willemientje,

without whom I'd never have finished.

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ABBREVIATIONS

PNPase	polynucleotide phosphorylase
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
AMP, GMP, UMP, CMP, IMP, XMP or pA etc.	the 5'-monophosphates of adenosine, guanosine, uridine, cytidine, inosine, xanthosine
ADP etc. or ppA etc. ..	their corresponding 5'-diphosphates
ATP etc. or pppA etc.	their corresponding 5'-triphosphates
dAMP etc.	the corresponding deoxy series
ApA etc.	adenylyl-(3'-5') adenosine etc.
ApdA etc.	adenylyl-(3'-5') deoxyadenosine
tRNA or sRNA	transfer or soluble RNA
mRNA	"messenger" RNA
polyA etc.	linear 3'-5' copolymer of adenylic acid etc.
poly AGUC etc.	linear 3'-5' copolymer of adenylic acid etc. in random sequence
NAD^+	oxidized nicotinamide-adenine dinucleotide
PMB	p-chloromercuribenzoate
NEM	N-ethylmaleimide
DTNB	5,5' dithio bis(2-nitrobenzoic acid)
PF	potassium ferricyanide
GSSG	oxidized glutathione
GSH	glutathione

BME	β-mercaptoethanol
tris	tris(hydroxymethyl)methylaminomethane
AS	ammonium sulfate
DEAE	diethylaminoethyl
QAE	diethyl-2-hydroxypropyl ammonium
SDS	sodium dodecyl sulfate
EDTA	ethylenediamine tetraacetate
TEMED	N'N'N'N'-tetramethylethylenediamine
pronase	streptomyces griseus protease
BSA	bovine serum albumin
RNase	ribonuclease
DNase	deoxyribonuclease
NDP, polyN	nucleoside diphosphate, polynucleotide

X° always refers to X° Celsius

SUMMARY

The polynucleotide phosphorylase from Azotobacter vinelandii has been shown to undergo a reversible oxidation-reduction and this discovery has led to the development for the first time of a reproducible procedure for the purification of the enzyme in high yield. The pure enzyme preparation contains a single protein species which can be dissociated into sub-units. Certain of the physical and catalytic properties of the pure enzyme have been determined, and compared to the previously published results for both A. vinelandii polynucleotide phosphorylase, and the enzyme from other bacterial species. Studies of the oxidized enzyme have identified the site of the reversible oxidation-reduction as certain sulfhydryl groups which are intimately involved in the catalytic properties of the enzyme. A. vinelandii polynucleotide phosphorylase, in common with the enzyme from other bacterial species, is stimulated by oligonucleotide primers in the polymerization of nucleoside diphosphates. This primer-requirement is not found in the freshly-prepared enzyme, but appears on storage of the otherwise-stable enzyme, and can be induced by limited proteolysis of polynucleotide phosphorylase by trypsin. The changes in primer-requirement are accompanied by changes in the electrophoretic properties of the enzyme. Finally, a

transnucleotidation activity has been for the first time unequivocally demonstrated in a polynucleotide phosphorylase and this is discussed in relation to the mechanism of action of the enzyme.

INTRODUCTION

a) General remarks

Polynucleotide phosphorylase (nucleoside diphosphate-polyribonucleotide nucleotidyl transferase; EC 2.7.7.8) was discovered in 1955 in Ochoa's laboratory (85,86,88) during studies on phosphate metabolism in Azotobacter vinelandii. They observed that crude extracts of this bacterium catalyzed the incorporation of [^{32}P] from orthophosphate into ADP (87), that is the exchange between the β-phosphate of ADP and orthophosphate. Shortly thereafter, the enzyme responsible was partially purified, and it was found that in addition to this exchange activity, the enzyme catalyzed the polymerization of nucleoside diphosphates to polynucleotides (86), and also the reverse reaction, that is, the breakdown of polynucleotides to nucleoside diphosphates, in the presence of orthophosphate (Table 1). A fourth activity, transnucleotidation, has also been reported (199). The enzyme was named polynucleotide phosphorylase (or PNPase, as I shall call it), by analogy with polysaccharide phosphorylase (86).

During early work it was found that the enzyme polymerized all the common nucleoside diphosphates, to give a product similar to natural RNA. During this early work it

Table 1

Activities of PNPase

a) Phosphorolysis:

$$(pN)_n + (n-2)P_i \rightleftarrows pNpN + (n-2)ppN$$

b) Polymerization:

$$(n)ppN \rightleftarrows (pN)_n + (n)P_i$$

c) Exchange:

$$ppN + {}^*P_i \rightleftarrows {}^*ppN + P_i$$

d) Transnucleotidation:

$$(pN)_n + (pN)_m \rightleftarrows (pN)_{n+1} + (pN)_{m-1}$$

was believed that PNPase was responsible for RNA synthesis *in vivo*, until it was established that it had no specificity with regard to the base sequence of the polynucleotide product. This theory was laid to rest with the discovery of RNA polymerase by Weiss and Gladstone (236) in 1959. In the meantime, PNPase had proven useful in the synthesis of RNA-like polymers of known base composition, which gave considerable insight into the physicochemical properties of nucleic acids.

b) Sources of PNPase

It was soon apparent that PNPase is ubiquitous, at least in bacterial species, and therefore must play an essential role in bacterial metabolism. Present theories center around the ideas that it may regulate the level of nucleoside diphosphates *in vivo*, or, that it is responsible for the degradation of RNA, especially "messenger" RNA. More recent work has been concentrated on the purification of the enzyme from various sources, with the intention of elucidating the reaction mechanism, as well as the structure of the enzyme protein itself. Grunberg-Manago's group in Paris have concentrated on the PNPase of *Escherichia coli*, Singer at NIH, and Fitt at the University of Ottawa, on *Micrococcus luteus*, and several workers have published results on *Clostridium perfringens* and *A. vinelandii*. Fitt's group has more recently

concentrated on PNPase from halophilic bacteria and from animal sources.

There have been no comprehensive review articles on the subject of PNPase for several years, and I think it useful to recapitulate the early work on the subject in the light of more recent data. In this introductory section then, I will attempt to give a comprehensive view of present knowledge about PNPase of A. vinelandii with the data obtained from other organisms.

i) Procaryotic organisms

Polynucleotide phosphorylase appears to be ubiquitous in bacteria (Table 2). The only microorganism tested in which it appears to be absent is Cl. acetobutylicum (27), but these authors also could not detect it in L. arabinosus, a result later found to be erroneous (217). Moreover, the PNPase in Clostridia appears to be much less active than in most other bacterial species (27). The tabulation shown, by no means proves the universal occurrence of PNPase. In many cases only one activity was tested, and certainly the exchange activity could be due to a series of other enzymes acting in concert. It also follows that the existence of a supposed PNPase cannot be considered as finally proven unless it has been purified. Conversely, where the enzyme is not found, it may be masked by other enzymes present in the crude extract. In fact, PNPase has been significantly

Table 2

Occurrence of PNPase in procaryotic organisms

Bacterial Species	Reference
Alcaligenes faecalis	27
Anacystis nidulans	33, 180
Azotobacter vinelandii (agilis)	86
Agrobacterium tumefaciens	230
Bacillus cereus	27, 205
Bacillus megaterium	167
Bacillus subtilis	14
Chlorella pyrenoidosa	180
Clostridium acetobutylicum	27
Clostridium kluyverii	27
Clostridium perfringens (welchii)	57
Corynebacterium diphtheriae	27
Escherichia coli B	27, 143
Escherichia coli Q13	222
Escherichia coli 1113B	36
Escherichia coli Crookes	27
Eubacterium sarcoginosenum	227
Halobacterium cutirubrum	173
Hydrogenomonas facilis	27
Lactobacillus arabinosus (plantarum)	217
Micrococcus luteus (lysodeikticus)	6
Mycobacterium avium	2
Mycobacterium phlei	27
Mycobacterium smegmatis	38
Neisseria meningitidis	118
Pseudomonas aeruginosa	208
Salmonella typhimurium	40
Staphylococcus aureus	27
Streptococcus faecalis	1
Streptococcus lactis	27
Streptococcus haemolyticus	27

purified from only four sources, A. vinelandii, E coli, M. luteus, and Cl. perfringens, and most of this discussion will be based on these enzymes. I should mention that PNPases isolated from these sources differ from each other in many of their properties and even PNPases isolated from different mutants of the same bacterium, e.g. E. coli, are radically different from that of the wild strain, as we shall see later.

ii) Localization in procaryotes

When PNPase is extracted from bacteria without regard to the preservation of subcellular structures, it is found in the soluble fraction. However, when care is taken in the isolation of ribosomes, the greatest part of the PNPase activity can be located in the ribosomes of Ps. aeruginosa (31,208). PNPase has also been reported in E. coli ribosomes (233). In the case of E. coli, the PNPase activity is higher in the 30 S ribosomes than in the 50 S ribosomes. When the Mg^{++} concentration is manipulated so that the 30 S and 50 S ribosomes associate to give the 70 S ribosome, the PNPase activity is inhibited, more than can be attributable to the change in Mg^{++} concentration alone (124). Very little PNPase activity can be detected in the polysomes. In S. faecalis most of the PNPase activity can be found in the cell membrane (1), much less in the ribosomes, and very little in the soluble fraction. Similarly PNPase of

H. cutirubrum is associated with the membrane fraction (173) and can be solubilized by brief sonication. Similar results have been reported for the blue-green alga A. nidulans (33). Unfortunately, no systematic study has been carried out to determine whether PNPase is in general associated with sub-cellular structures or whether the examples cited are simply exceptions to the rule. It is possible that the relatively violent techniques generally used for isolation of PNPase release the enzyme into the soluble fraction.

The activity of PNPase in bacteria varies with the stage of growth. In E. coli B, the polymerization activity is more than 1½ times higher in the early logarithmic growth phase than in the middle to late log. phase (83). In A. faecalis, the exchange activity is almost four times higher in early log. phase than in late log. or stationary phase (27). Similar results have been reported for A. vinelandii (27). There is also a suggestion that a parallelism exists between levels of PNPase and RNA synthesis in vivo (142,241).

iii) Higher organisms

Some justifiable doubt existed until recently as to whether PNPase occurs in higher organisms. Early reports indicated the presence of PNPase activity in guinea pig liver nuclei (105,110) and also in extracts of spinach leaf (27). Since that time PNPase has been reported in a variety

of multicellular systems (Table 3). In most of these cases, either only one activity was tested, or only relatively crude preparations were used, or, most often, specificity was not proven. Moreover, in multicellular organisms one cannot discount the possibility of bacterial contamination or infection, which could lead to a false indication of PNPase activity. Some authors have taken the existence of PNPase in animal tissues as proven without characterization (30,175). One cannot but discount these results as proof of existence of the enzyme.

Recent work (190) has established that PNPase is present in the mitochondrial inner membrane of calf, chicken, guinea pig, rabbit and rat liver, rat heart, kidney, spleen and muscle, and goldfish muscle. Studies of the intracellular localization of the enzyme in rat, and to a more limited extent guinea pig liver, suggest that it may also occur in loose association with the nucleus (68,188) but it appears to be absent from other subcellular fractions. The rat and guinea pig liver enzymes have both been purified to some extent as particulate enzymes, and rat liver mitochondrial PNPase, in particular, has been obtained free from phosphatase contamination. The specificity of the rat and guinea pig enzymes for nucleoside diphosphates as products of the phosphorolysis of polyribonucleotides has been unequivocally demonstrated. These authors have thus accounted for all the objections to previous claims. The

Table 3

Occurrence of PNPase in higher organisms

Source	Reference
Ascaris lumbricoides (earthworm)	62
Barley sprouts	122
HeLa cell nuclei	99
Human sperm	97
Human urine	98
Guinea pig liver nuclei	105, 110
Millet sprouts	122
Rat brain	161
Rat epithelioma	240
Rat liver	30, 175
Spinach leaf	27
Wheat roots	123
Wheat sprouts	122
Guinea pig liver nuclei	68, 188
Rat liver nuclei	68, 188, 190
Guinea pig liver mitochondria	189
Calf liver mitochondria	190
Rabbit liver mitochondria	190
Rat liver mitochondria	190
Rat heart mitochondria	190
Rat kidney mitochondria	190
Rat spleen mitochondria	190
Rat muscle mitochondria	190
Goldfish muscle mitochondria	190

animal PNPases are quite unlike the bacterial enzymes, in that they possess no polymerization activity.

c) Purification

Since practically every paper on the properties of PNPase also includes a new method of purification, it would require a large book to describe all these methods and their modifications. I shall attempt to be brief and rather selective in the outline of the problems involved in purification. The only PNPases which have been extensively purified are, in estimated ascending order of final purity (based on values of specific activity), those from Cl. perfringens, M. luteus, E. coli and A. vinelandii.

i) Cl. perfringens

I mention Cl. perfringens PNPase only because some of its properties, which I shall discuss later, are of extreme interest. Dolin (58) originally purified the enzyme to a specific activity of 290 ADP units/mg (with polylysine) with a yield of 11% of the crude extract units. His procedure involved, in turn, ammonium sulfate, protamine and again ammonium sulfate precipitation, followed by calcium phosphate gel fractionation and zone electrophoresis. The final enzyme was absolutely dependent on polylysine for ADP-incorporation activity. Fitt and Wille (69) improved the procedure, and obtained a specific activity of 355

units/mg with a yield of 15%. The steps in their procedure were ammonium sulfate, protamine fractionation, DEAE-Sephadex chromatography, G-200 gel filtration, and a second DEAE-Sephadex chromatography. The final product retained some activity in the absence of polylysine, and could be detected on polyacrylamide gel electrophoresis by the correspondence of a band of protein and enzymic activity. However, the enzyme was far from pure.

ii) M. luteus

The most extensive body of literature on PNPase concerns the enzyme isolated from M. luteus (formerly lysodeikticus). The original attempts at purification were by Beers (8), but the first serious attempts were by Singer's group (196,213). After continuous modifications and additions to the procedure, they were able to obtain a PNPase with a specific activity of 397 units/mg in 5% yield (131). Their final procedure involved lysozyme lysis, ammonium sulfate fractionation, DEAE-cellulose chromatography, zinc precipitation, Sephadex G-100 and G-200 gel filtration, and microgranular DEAE-cellulose chromatography. The enzyme is claimed to be 95% pure on the basis of polyacrylamide gel electrophoresis (127), which revealed only one protein band other than the enzyme band. On occasion the enzyme was pure, but in general a further hydroxylapatite chromatography step was necessary to obtain pure

PNPase (128). This preparation had a phosphorolytic specific activity of 240 units/mg, and an ADP-incorporation specific activity of 2,000 units/mg, and contained no extraneous protein bands on gel electrophoresis. Some pure preparations had specific activities as low as 180 phosphorolysis units/mg or 1,000 ADP incorporation units/mg.

iii) E. coli

PNPase from E. coli B has also been extensively purified. Littauer and Kornberg (143) originally purified this enzyme to 28 ADP-incorporation units/mg by protamine and ethanol fractionation, in 50% yield. Kimhi and Littauer (125) were eventually able to improve on this by a procedure involving ultracentrifugation, ammonium sulfate precipitation, autolysis, acetone fractionation, DEAE-Sephadex chromatography and G-200 gel filtration, to obtain an enzyme with 436 phosphorolysis units/mg in 10% yield. The preparation was, however, judged impure by sedimentation in the ultracentrifuge and also contained nuclease activity. The best published purification procedure for E. coli PNPase is that of Williams and Grunberg-Manago (238). The procedure involved ammonium sulfate fractionation, protamine precipitation, DEAE-Sephadex and DEAE-cellulose chromatography, and Sephadex G-200 gel filtration. The preparation contained no phosphatase or kinase activity, and negligible nuclease activity. It however was only some 60%

pure by Schlieren ultracentrifugation, although at least part of the impurities may have been PNPase isoenzymes. This purification method is referred to in succeeding papers from the laboratories of Grunberg-Manago (90) and Thang (216). In these papers it is suggested, but never specifically stated, that the enzyme is free from contaminating proteins. Lehrach et al. (137), using the Williams procedure, obtained an enzyme with a single protein species, and published photographs of polyacrylamide gels which would support a claim of absolute purity. There was no mention in the literature of how this final purification was accomplished, except for a reference to unpublished results (90), nor any mention of the specific activity of the pure enzyme, other than a claim of 1000 phosphorolysis units/mg for an admittedly impure preparation (90). Judging from the literature, I personally am convinced that the enzyme has been purified absolutely, but I must admit that there is valid reason for doubt.

iv) A. vinelandii

Although PNPase was originally detected in extracts of A. vinelandii in 1955, serious attempts at purification were not made until 1961, by Ochoa and Mii (166). Their method is shown in Table 4. Note that, although the units have been recalculated to the system I use, defined in the "Methods" section, they are still not strictly comparable,

Table 4

Purification of PNPase from A. vinelandii I
after Ochoa and Mii (166)

Enzyme fraction	Total protein	$\frac{A_{280}}{A_{260}}$	Phosphorolysis	
			Total units	Specific activity
crude extract from 1 Kg cells wet weight	101,000	0.60	405,000	4.0
ammonium sulf.	31,000	0.60	230,000	8.0
ethanol fraction	5,400	0.60	168,000	32.0
calcium phosphate gel eluate	400	0.65	71,200	180
protamine and ammonium sulf.	54	0.91	48,800	904
hydroxylapatite chromatography	23	0.92	32,200	1400
chromatography repeated	14	0.93	27,700	1980

because the assay conditions are quite different from mine (e.g. phosphorolysis measured by a pyruvate kinase-coupled system, at pH 7.4). Judging from their results for the chromatography of the enzyme on hydroxylapatite, the enzyme is some 90% pure, but contained some impurities, notably a polynucleotide and possibly a nuclease. Unfortunately, the method was not reproducible, even in the hands of the original authors (203). Thang developed an alternate procedure for purification, which is claimed to be reproducible (214). A summary is shown in Table 5. This enzyme contains no activity other than PNPase, and exhibits only one peak during sucrose density gradient ultracentrifugation. There are, however, two protein bands on polyacrylamide gel electrophoresis, only the more intense, slower-moving one having PNPase activity. No estimate is given of the percentage of PNPase protein in the purified preparation. Moreover, this method yields only 9% of the units in the crude extract, which is comparable to the yields of pure enzyme from other sources, but as we shall see, can be improved for A. vinelandii.

v) Relative yields

In terms of phosphorolysis units, assuming a wet weight:dry weight ratio of 5:1, M. luteus yields 12 units/g cells (wet weight) (131), E. coli, 74 units/g (238), and A. vinelandii, 180 units/g (214). Thus A. vinelandii is by

Table 5

Purification of PNPase from A. vinelandii II
after Thang (214)

Enzyme fraction	Total protein	$\frac{A_{280}}{A_{260}}$	Phosphorolysis	
			Total units	Specific activity
crude extract from 100 gm cells dry weight	34,000	0.5	89,800	2.64
ammonium sulf. 34-46%	12,300	0.63	111,000	9.0
calcium phosph.	634	0.55	57,900	.92
ammonium sulf. 42-55%	157	0.93	49,500	315
DEAE-Sephadex A-50	32	0.95	25,300	790
DEAE-cellulose	6.4	0.96	8,830	1380
ammonium sulf. concentration	5.5	0.95	8,060	1480

far the best source of PNPase. The best purification steps, in terms of both yield and purification, are those involving the various Sephadexes. DEAE-cellulose chromatography, or calcium phosphate gel generally give good purification with poor yields of units. Zinc chloride precipitation and hydroxylapatite chromatography seem to be powerful methods in individual cases but are not generally employed for all the different PNPases. All the other methods described are not especially effective, but are necessary for the purification of relatively crude preparations.

A. vinelandii PNPase has the highest specific activity in the pure state (1,480 units/mg), followed by E. coli (1000 u/mg) and M. luteus (400 u/mg). However, the pure A. vinelandii PNPase is contaminated by nucleic acid (214) and has a low A_{280}/A_{260} ratio (0.95) unlike the E. coli (238) and M. luteus (131) enzymes which are free of nucleic acid. Nevertheless, because of its high yield and specific activity, I chose to concentrate on A. vinelandii PNPase for the experiments in this work.

d) Assays of PNPase activity

Various assays have been developed for the measurement of PNPase activity. Grunberg-Manago et al. (88) in their original paper described assay procedures for all three activities of PNPase. In the first case, exchange, the enzyme was incubated with NDP, $MgCl_2$ and $[^{32}P_i]$; then

the reaction was stopped with trichloroacetic acid. The extent of reaction was measured by extracting the unreacted inorganic phosphate and measuring the specific radioactivity of the organic phosphate. The extent of reaction is then given by the expression

$$\mu\text{mol phosphate incorporated} = \frac{\text{cpm incorporated } (\mu\text{mol phosphate} + \mu\text{mol NDP})}{\text{cpm phosphate}}$$

The polymerization activity was assayed by incubating the enzyme with NDP and $MgCl_2$, precipitating the polyN with trichloroacetic acid, and measuring the orthophosphate released in the supernatant. These authors also described an assay procedure for the phosphorolytic activity. This involved the same procedure and workup as their exchange assay, substituting either RNA or synthetic polyN for NDP. The μmol of phosphate incorporated in this case is of course directly given by the cpm incorporated. All other assays for PNPase activity are with a few exceptions, modifications of the above procedures.

Modifications to the phosphorolysis assay include pyruvate kinase-coupled systems in which NAD^+ formation is measured spectrophotometrically (166,239), or pyruvate formation is measured colorimetrically (94). A very recent modification for phosphorolysis and exchange assays involves the specific precipitation of inorganic phosphate, and measurement of the radioactivity of organic phosphate in the supernatant (45). Because of the greater interest in the

ADP-incorporation assay, several independent methods of assay have been developed. The original Grunberg-Manago procedure has been automated (29). The activity has been measured by following the change in viscosity of the reaction mixture with time (8) but this is complicated by anomalous viscosity changes during the reaction (102). Since there is a hypochromic effect at 260 nm, when mononucleotides condense to form polynucleotides, one can use this effect for measuring activity (24). Conversely, one may follow the hyperchromic effect at 285 nm (238). The simplest modification, and the one that I use, involves the precipitation of radioactive polynucleotide product, collection on a glass-fibre filter, and direct counting of radioactivity (143). Two other assays, used occasionally by Singer's group, are based on the arsenolysis of NDP (194, 185) or polyN (203).

e) pH optimum

i) Polymerization

Early reports indicated that PNPase from M. luteus had a fairly basic pH optimum for the polymerization reaction, regardless of the nucleoside diphosphate used (8, 169). Further studies showed that this value depends, in part, on the concentration of salts and Mg^{++} in the reaction mixture: at high levels of Mg^{++} the optimum is lower in the

absence of salts, than in their presence; at low Mg^{++} levels salt has no effect (9). Depending on the conditions, the optimum may be as high as pH 10 (6). It is generally accepted that there is a plateau of activity between pH 9.0 and pH 10.0 (169,196). Of some interest is the fact that high pH shifts the equilibrium in favour of polymer formation (6), and has a stimulating effect on reaction rate, especially for guanine-containing copolymers (22). In addition, the non primer-requiring M. luteus PNPase has a slightly lower pH optimum than the primer-requiring form and the latter has a lower primer requirement at high pH than at lower values (196).

PNPase from other sources has not been as extensively studied with respect to pH optimum. A. vinelandii PNPase was reported to have a plateau of optimum activity between pH 7.5 and pH 9.0; at pH 6.4 the activity was only 30% of that at the plateau (88). Later work showed that the optimum pH was 9.5 (214). Similarly the E. coli enzyme was reported to have a pH optimum at pH 7.4 (143), later found to be approximately pH 10.0 (238). It would appear that the more purified the enzyme, the more the different PNPases resemble each other in pH optima (214). Further, E. coli PNPase can exhibit a primer requirement at low pH, which diminishes at higher pH (238), similar to the effect shown for M. luteus. Perhaps by analogy with the micrococcal enzyme, the E. coli enzyme has a higher optimum pH for GDP

polymerization than for ADP (218). PNPases from other organisms appear to have similar pH optima; H. cutirubrum PNPase has an optimum between pH 8.2 and pH 9.0 (173), that from N. meningitidis between pH 8.7 and pH 9.3 (118), and E. coli Q13 shows a plateau above pH 8.0 (113).

Finally, there is the PNPase from Cl. perfringens, which shows a plateau between pH 7.0 and pH 9.0. In the presence of salt the optimum is above pH 9.0, and in the presence of polylysine as activator there is a sharp optimum at pH 8.2 (64). The latter effect may, however, be a property of the enzyme-activator complex, rather than of the enzyme itself.

ii) Other activities

Other activities of PNPase have not been as extensively studied as the polymerization reaction. A. vinelandii has a sharp optimum for exchange at pH 8.1, with only 20% of this activity at pH 6.4, the activity falling rapidly above pH 8.6 (124). M. luteus has an optimum pH of 8.7 for the exchange, phosphorolysis, and arsenolysis of polynucleotides (203). However, the arsenolysis of nucleoside diphosphates is 30% faster at pH 8.2 than at pH 8.7 (194). Similar values have been reported from other sources: E. coli, phosphorolysis, pH 8.1 - 10.0 (83); E. coli Q13, phosphorolysis, pH 8.0 - 9.0 (223); A. vinelandii, phosphorolysis, pH 7.5 (166) (note that the assay procedure is a coupled

system so this value may be in error); H. cutirubrum exchange, pH 8.2, phosphorolysis, pH 9.8 (173). Finally, guinea pig liver PNPase, which was earlier reported to have a pH optimum for phosphorolysis around pH 7.0 (110) has been found to have an optimum between pH 8.6 and pH 9.2, depending to some extent on the Mg^{++} concentration (188).

f) Temperature optimum and stability

For E. coli PNPase, the optimal temperature for ADP and GDP polymerization is about 60°, while that for CDP and UDP is between 45° and 55° (147). The optimal temperature for polyA and polyC phosphorolysis is around 60°, and between 50° and 55° for polyU. For M. luteus PNPase, the optimum temperature for ADP polymerization is 45°, and for UDP polymerization, 36° (23). PolyU phosphorolysis is optimal at 46°. E. coli PNPase is heat-stable below 55° and the enzyme is partially protected from inactivation at higher temperatures by nucleoside diphosphates, polyribonucleotides, and 3'OH oligonucleotides, but not by nucleoside mono- or tri-phosphates, polyvinylsulfate, calf-thymus DNA, or 3'phosphate oligonucleotides (147). Conversely, heat-inactivation of M. luteus PNPase is accelerated by nucleoside diphosphates, especially GDP, but partially prevented by polynucleotides (23). Thus, M. luteus and E. coli PNPases differ significantly in their response to elevated temperatures.

g) Metal ion requirement

In general PNPase requires Mg^{++} as a cofactor for all its catalytic activities. No reaction occurs in its absence. It was generally accepted that no other metal ion could replace Mg^{++} (83) and, in fact, such ions as Mn^{++} (118), Cu^{++}, Zn^{++} and Ba^{++} (169,196), inhibited polymerization in the presence of Mg^{++}. However, Beers had earlier shown with the M. luteus enzyme that Mn^{++} could support polymerization, to the extent of 47% of that formed with Mg^{++}, and that Ca^{++} and Ba^{++} were active to lesser extents (8). Much later, it was shown with E. coli PNPase, that high molecular weight polyG could be synthesized at high temperature six times faster using Mn^{++} as ccfactor rather than Mg^{++} (218). This could not be repeated with the A. vinelandii enzyme (214). However, the question of metal ion requirement was reopened and it was found that other divalent cations could replace Mg^{++} (3). With E. coli PNPase in the phosphorolysis reaction Mn^{++} was nearly as effective as Mg^{++}, with Co^{++}, Cd^{++}, Zn^{++} and Cu^{++} being progressively less effective. Ca^{++} was ineffective. In the polymerization reaction Mg^{++} was best, followed by Mn^{++} at low concentrations, then Co^{++}, Zn^{++} and Cd^{++}. It was confirmed that Mn^{++} was inhibitory in the presence of Mg^{++}. Similar results were given for the A. vinelandii and M. luteus enzymes. In the exchange reaction, Mn^{++} was 40% as effective as Mg^{++}, and was inhibitory in the presence of

Mg^{++}, whereas Cd^{++}, Co^{++}, and Zn^{++} were still less effective but slightly stimulatory in the presence of Mg^{++} (125). Ca^{++} and Ni^{++} were ineffective. Cl. perfringens PNPase appears to have an absolute requirement for Mg^{++} (96). Thus for normal PNPase from most sources Mg^{++} is the activating ion.

There is, however, a group of PNPases which require Mn^{++} for some of the reactions. The "heavy" PNPase of E. coli Q13 requires Mn^{++} for the polymerization reaction (113), but Mg^{++} for the very low phosphorolysis activity (214). The low molecular weight PNPase present in this organism is, however, Mg^{++}-requiring and catalyzes only phosphorolysis (223). Similar results were found in E. coli 1113B (36) and it was also shown that the Mg^{++} was partially effective in these two mutants in the polymerization reaction, as was Mn^{++} in the phosphorolysis reaction. Finally, there is the very low molecular weight PNPase of H. cutirubrum which requires Mn^{++} for all three activities, although Mg^{++} stimulates the polymerization in the presence of Mn^{++} (173).

h) Specificity

i) Substrate specificity

PNPase, by definition, catalyzes the reversible polymerization of ribonucleoside diphosphates to

polyribonucleotides. Thus, for the polymerization reaction, it employs as substrates the four common nucleoside diphosphates ADP, CDP, UDP and GDP, although the last only with difficulty, except under special conditions. All the early reports on the enzyme from various sources reiterate this conclusion, e.g. (88). Further, PNPase catalyzes not only homopolymerization of the individual nucleotides, but also heteropolymerization of mixtures of the nucleotides, giving a polynucleotide similar in base composition to the original nucleotide mixture. The copolymers have a random base-sequence, so PNPase is not specific in the sense that RNA or DNA polymerase are. Again, copolymers of GDP and one of the other nucleotides are synthesized only with difficulty, but polyAGUC of varying composition is readily synthesized. I shall return to the question of GDP polymerization at a later point.

PNPase is absolutely specific with respect to the esterification of the nucleoside: only nucleoside-5' diphosphates act as substrates; nucleoside mono- and triphosphates are not polymerized, nor are 3'esterified nucleosides (81). Further, it is specific for the phosphate moiety, since such analogues as adenylyl-5'methylene diphosphonate (192), and UMPS or AMPS (in which the terminal phosphate is replaced by sulfate (81)), are not substrates. The attachment of the base to the sugar is also important, since only the naturally-occurring β-anomers are active;

α-nucleoside diphosphates are inactive as substrates (164). Some base is a necessary component of the substrate; ribose-5' pyrophosphate is not a substrate (165). In general, proof that a given enzyme is a PNPase requires unequivocal demonstration of specificity for NDP as substrate for the polymerization or product in phosphorolysis.

So much for absolute specificity. All the compounds listed in Table 6 do act as substrates for PNPase, even though in many cases they react only to a very limited extent and, in fact, some are highly inhibitory. Further, I do not distinguish at this point between compounds which act as substrates in the exchange reaction but not in the polymerization reaction, nor whether they are good or poor substrates.

Although, in early work it was assumed that PNPase was specific for the ribose moiety, since it did not employ β-D-arabinosyl UDP (153), or deoxyribosyl TDP (81) as substrates, recently it has been found that dADP can act as a substrate for a limited polymerization (more exactly an elongation of a pre-existing ribo-oligonucleotide) (9,10,20, 121). In addition, compounds in which one of the hydroxyl groups on the ribose is esterified (e.g. 2'0-methyl NDP) are substrates for extensive heteropolymerization with the normal nucleotides, but not for homopolymerization (117, 182).

The problem of specificity becomes more complicated

Table 6

Substrates for PNPase

Substrate	Reference
a) Polymerization substrates	
ADP	86
UDP	86
CDP	86
GDP	86
IDP	86
XDP	226
pseudo UDP	185
dihydro UDP	209
S^4 UDP	193
rTDP	93, 200
3-iso ADP	140, 155
dADP	9, 10, 20, 121, 50
5-F UDP	152
5-Br UDP	152, 153, 7
5-Cl UDP	152, 153, 7
5-I UDP	152, 153, 7
5-OH UDP	152
5-methyl UDP	152
5-ribosyl UDP	152
1-methyl ADP	91
1-methyl GDP	174
6-hydroxyethyl ADP	154
6-aza CDP	206, 207

Table 6 (cont'd)

Substrate	Reference
6-thio IDP	34
2-thio UDP	138
8-aza GDP	141, 205
4-methyl CDP	25
4,4-dimethyl CDP	25
N'-methyl UDP	209
N'-methyl ADP	92
N^6-methyl ADP	91
N^6,N^6-dimethyl ADP	91
2'0-methyl CDP	117, 182
2'0-methyl ADP	117, 182
2,6-diaminopurine riboside 5'diphosphate	112
2-aminopurine riboside 5'diphosphate	232
b) Phosphorolysis substrates	
rRNA	79
sRNA	202
mRNA	2
TMV RNA	79
TYM RNA	79
phage-induced RNA	191
poly 4-thio UDP	187, 193
aminoacyl tRNA	unpub. res. in 121

when we study the nature of the base. Firstly, the base must have some sort of groups attached to the nucleus of the base since ribosylpurine-5'pyrophosphate is not a substrate (5). Further than this it is difficult to draw conclusions, since, as we can see from Table 6, many substituted purines and pyrimidines act as substrates. The question has recently been resolved by Michelson's group (119), in a paper which is more properly discussed under the heading of "Inhibition." Suffice it to say that all 5'ribonucleoside diphosphate derivatives can act in some way as substrates for PNPase, subject to certain steric restrictions.

With respect to the phosphorolytic activity, PNPase can, of course, phosphorylize any compound which it can synthesize. For example, DNA is not phosphorylized, nor are oligonucleotides with a 3'-terminal phosphate or a 2' 3' cyclic phosphate, although this does not apply to long-chain polynucleotides (79). PolyC-N-oxide is not phosphorylized (51). Conversely, oligonucleotides with or without a 5'-terminal phosphate are readily phosphorylized. Although PNPase polymerizes nucleoside diphosphates without any added primer (de novo chain formation), it cannot reverse this step. The products of phosphorolysis are always mononucleotides and dinucleotides, which are resistant to further phosphorolysis, as are dinucleoside monophosphates (134). An important requirement is that the polynucleotides must be single-stranded, i.e. non hydrogen-bonded. Thus sRNA under

normal conditions is only partially phosphorylized. When the double-stranded structure is destroyed by chemical or physical means, sRNA is readily phosphorylized (220). For this same reason polyG is quite resistant to phosphorolysis, because under normal conditions it is multi-stranded (71). Thus polyG is phosphorylized readily only under conditions which inhibit the formation of multi-stranded complexes, e.g. high pH, high temperature, high salt concentration, presence of Mn^{++} (218). Copolymers of GDP and other NDP derivatives are also resistant to phosphorolysis, the resistance being proportional to the GMP content (79). These results can explain the difficulties in polymerizing GDP, except in conditions which minimize hydrogen-bonding e.g. the conditions above, presence of primer, presence of other nucleoside diphosphates.

ii) Inhibitors

Inhibitors of PNPase can be arbitrarily divided into three groups: (1) "non-specific" inhibitors, such as inorganic ions or prosthetic group-binding reagents; (2) Product or product-analogue inhibitors, such as certain oligonucleotides; and (3) substrate analogue inhibitors. Typical examples of these three classes of inhibitors are listed in Table 7.

The first term is perhaps a misnomer, since the group includes many compounds which have very specific modes of

Table 7

Inhibitors of PNPase

Inhibitors	Reference
a) "non-specific" inhibitors	
urea	52, 102
tetracyclines	74, 75
polylysine	238
cysteamine	19
2-mercaptopropylamine	19
β-mercaptoethanol	19
Acridine orange	12, 15
Salts	9, 83, 88
Mg^{++}	9, 88
Mn^{++}	143, 225
phosphate	203
arsenate	203
$CuSO_4$	169, 170
$ZnCl_2$	169, 170
$BaCl_2$	169, 170
iodoacetate	169, 170
streptomycin	143
b) oligonucleotide inhibitors	
3'phosphate oligo N	43, 108
sRNA	73
poly G	22
Yeast RNA	116
Specific polymers	104
oligo N inhibition of RNA degradation	163

Table 7 (cont'd)

Inhibitors	Reference
ribosomes	36, 37
see also	10, 11, 14, 89, 103, 156, 196, 200
c) substrate analogue inhibitors	
dihydro UDP	209
dADP	46, 146
dCDP	146, 149
8-substituted purines	114
8-Br GDP	119
8-oxo GDP	119
6-methyl CDP	119
6-mercaptopurine 5'riboside diphosphate	119, 34
2,6-quinazolidinedione 1'riboside diphosphate	119
6-aza UDP	119, 204
6-aza CDP	207
3-methyl 6-aza UDP	120
8-aza GDP	206
6-mercaptopurine	34
6-thioinosine 5'diphosphate	34
GDP	22, 23
2'0-methyl NDP phosphonate	182
adenosine 5'methylene diphosphonate	192
9-β-arabinosyl ADP	146
1-arabinosyl 5'diphosphate	83
α-UDP	164
ppUp, ppAp	81, 153

inhibition, and which differ from each other in their action. In particular, urea appears to inhibit the chain-initiation step (52,102) and the enzyme is protected from inactivation by 3' hydroxy oligonucleotides. Various substituted tetracyclines inhibit by binding Mg^{++} (74,75). Similarly, polylysine may inhibit PNPases of the E. coli type by the same mechanism, but there remains some doubt (238). Various sulfhydryl compounds inhibit the activity (19), supposedly by interaction with the active site, but this has been denied by other authors. Acridine orange inhibits by binding the substrate at the 3'OH although this can be reversed by Mg^{++} (12,15). Salts, such as KCl and NaCl, are stimulatory at low concentrations, and inhibitory at high concentrations, but this effect is better discussed under the heading of "Kinetics" (9,83, 88). Similarly, Mg^{++} has a complex activating and inhibiting effect, intimately involved in the catalytic mechanism of PNPase (9,88). Mn^{++}, which can in some cases replace Mg^{++} as an activating ion, is also inhibitory in the presence of Mg^{++} (143,225). Phosphate and arsenate, of course, inhibit because they are products of polymerization or arsenolysis (203). It was reported that $CuSO_4$ and $ZnCl_2$ selectively inhibited CDP-polymerization, and $BaCl_2$ inhibited ADP-polymerization (169,170), but it must be remembered that these experiments were done with a modified trypsin-treated PNPase, and could not be confirmed

by others (196). Similarly, these authors reported inhibition by iodoacetate, where others found little or no effect of sulfhydryl-binding reagents (88,196). Streptomycin inhibits by selectively binding substrate, and its effect can be reversed by RNA (143). However, not all compounds which bind PNPase inhibit it. The most striking example is the fact that a PNPase-antibody complex is enzymatically active in immunodiffusion gel assays (228), although it is not specified whether there is partial inhibition. Similarly, PNPase is active when absorbed to nitrocellulose filters (219). It is clear that this list is by no means complete, and that many more compounds could be found, which would inhibit PNPase in a more or less specific manner.

Of greater interest, with respect to the mechanism of action of PNPase is the second class of inhibitors, i.e. oligo- and polynucleotides. This class again includes several different specific kinds of inhibition. For example, several authors have shown that 3' phosphate-ended oligonucleotides inhibit the polymerization reaction (43). This could be attributed to a form of product inhibition, in that the product analogue might bind the enzyme. 3' phosphate oligonucleotides also inhibit phosphorolysis, and this has recently been shown to be competitive with respect to substrate (43). Inhibition by polynucleotides which are resistant to PNPase is a general phenomenon.

sRNA (73) and polyG (22) which are resistant because of their secondary structures, or yeast RNA (3'P) (116), inhibit both polymerization and phosphorolysis. An interesting point in connection with this is that, although one may expect mixtures of polynucleotides which can form multiple-stranded structures (e.g. polyA + polyU) to be inhibitors for phosphorolysis, they are also inhibitors for polymerization (104). Thus, polyU inhibits ADP polymerization, and polyA inhibits UDP polymerization. The explanation put forward is that chain-initiation is much faster than elongation, and that the inhibitory polymer binds the newly synthesized oligonucleotide, detaching it from the enzyme. Eventually, the inhibitory polynucleotide is removed by this process and the mixture exhibits normal kinetics. Alternatively, the inhibition is due simply to binding of the polymer to the enzyme. Possibly the inhibition of RNA degradation by oligonucleotides may be part of this phenomenon (163), but these authors suggest that the inhibition is due to the 3'OH oligonucleotides being more slowly phosphorolyzed by PNPase. An interesting form of inhibition (or more correctly, protection) is that by ribosomes in the autodegradation of mRNA (36) or the phosphorolysis of polyU (37). In this case it has been shown that PNPase phosphorolyzes the polynucleotide only to the point of attachment with the ribosome.

The third class of inhibitors is the most interesting with respect to the mechanism of PNPase. Of the substrate analogues listed in Table 7, part 3, most are strongly inhibitory, and have been usually considered to be totally inactive as substrates. Until recently no systematic theory had been developed to account for their inactivity. Michelson's group has developed an explanation for PNPase inhibition of this type, based on steric considerations (119) and the following discussion is taken from their work. They classify nucleotide analogues into three classes: those that behave in a manner similar to natural nucleotides (viz. Table 6, ψUDP, 5-X-UDP, 6-hydroxyethylADP, 8-azaGDP and others); those that are neither good substrates nor strong inhibitors (dihydro-UDP and dADP); and those that are strongly inhibitory (in general 8-substituted purines and 6-substituted pyrimidines, although 8-azaGDP does not fall in this class, whereas 6-mercaptopurineriboside 5' diphosphate does). "The salient features of this class of inhibitors" is that their [substituent] "groups restrict the rotation around the glycosyl bond of the corresponding monomers and polymers, in a manner which makes the <u>syn</u> conformation energetically more favourable than the <u>anti</u> conformation." That is, for PNPase activity, the substrate must have "close aposition of the 2'-hydrogen of the ribofuranose and either the C-8 hydrogen of purines or the C-6 hydrogen

of pyrimidines." This incidentally can also account for inhibition by other inhibitors of this general class, e.g. dNDP, 2'O-methyl NDP, αUDP, etc. An inferred corollary is that all these inhibitors are actually substrates of PNPase, but in some cases are such poor substrates that polymerization (or phosphorolysis of their polymers) does not actually occur. It should be noted that only the 5' riboside diphosphates exhibit this sort of inhibition, since, for instance, 6-azaUMP is not an inhibitor (206). The other substrate-analogue inhibitors listed, e.g. ppUp (81) and adenosine 5' methylene diphosphonate (192), must inhibit because of other steric or chemical considerations.

iii) Stimulators

Stimulators of PNPase activity can be conveniently divided into two classes; oligonucleotide "primers" and others. The second class is again a potpourri of many different kinds of compounds. For instance, an unidentified heat-stable activating factor for exchange activity has been reported in E. coli (125,143). The membrane-bound PNPase of S. faecalis is stimulated by the soluble fraction (1), possibly an oligonucleotide. Various salts stimulate PNPase (7,8,75,103) at low concentrations, whereas at high concentrations they are inhibitory. In the case of polymerization the salts appear to stimulate

the binding of substrate to enzyme (9), whereas for phosphorolysis the stimulation is explained as a disruption of hydrogen-bonding (secondary structure), at least for naturally-occurring RNA (79). Acridine orange, which is also inhibitory at high concentrations, stimulates phosphorolysis at low concentrations, due to a "neutralization of negative charges" on the polymer (12). Cystamine and bis-(2-aminopropyl) disulfide are said to stimulate polymerization, by an unspecified mechanism (19). ATP stimulates polyAG formation (91) while glycine, asparagine, lysine and histidine stimulate polyAGUC formation and even affect the purine-pyrimidine ratio in the product (54). A series of Russian papers suggest that various polyamino acids and polybases specifically stimulate polymerization and exchange in E. coli PNPase (viz. 150). Thus polylysine, protamine and spermine stimulate ADP exchange (but inhibit others), polyphenylalanine stimulates UDP polymerization and exchange, and poly-L-proline and L- (but not D-) lysine stimulate CDP polymerization.

These last results may provide a point of similarity between "normal" PNPases and the special case of Cl. perfringens PNPase. This PNPase is atypical in that it is greatly stimulated by polybases in the ADP- and GDP-incorporation assays (57,58). Some purified fractions were obtained which completely lacked phosphorolytic and CDP- or UDP-incorporation activities (55,96,133), and even

in crude extracts polybases inhibited these last activities. Polylysine, polyornithine, and polyvinylamine were all effective as activators for stimulation of ADP incorporation, and could be partially replaced by inorganic salts or bases, such as L- or D-lysine, and diethyl- or triethylamine (64,69,70). The stimulation increases with increasing chain length in the polylysyl peptide series, reaching a maximum at thirty residues (70). The effect is explained as a relatively non-specific ionic effect on the enzyme itself (69).

iv) Primers

The most interesting stimulators of activity are oligonucleotides. It was observed that relatively pure A. vinelandii PNPase showed autocatalytic kinetics in the incorporation assay (156), i.e. a lag phase, and that this could be eliminated by polynucleotides. This was confirmed for M. luteus (14) and E. coli PNPase (238). In the case of stimulation by polynucleotides the effect was quite specific, in that polyA and polyC "prime" their own synthesis; polyC primes the synthesis of a variety of polynucleotides, but polyC synthesis is inhibited by any other polynucleotide. This is perhaps a special case of a more general phenomenon, that is, priming by oligonucleotides (197,198). For instance, early commercial ADP preparations were found to contain a stimulator identified

as short-chain oligonucleotides (14). I must emphasize that the term "primer," as used here, refers not to a template, but rather to a stimulating oligonucleotide which may or may not be incorporated into the product. In early work it was reported that both 3'OH oligonucleotides (which are incorporated into the product), and 3' phosphate oligonucleotides (which are not) can act as primers (108). The reason for this remained unexplained until recently, and it now seems likely that priming by 3' phosphate oligonucleotides was actually due to contamination by 3'OH oligonucleotides (90). This being the case, priming must therefore involve the elongation of the oligonucleotides, and the stimulating effect is due to the fact that the chain initiation step (condensation of two DNP molecules) is much slower than elongation. Nevertheless, oligonucleotides stimulate de novo synthesis, possibly by maintaining the enzyme in an active conformation. Incidentally, native freshly-prepared PNPases from the usual sources are not stimulated by primer except in special cases, but E. coli (216) and M. luteus PNPase (P. S. Fitt, private communication) develop a primer requirement very quickly on storage. This primer requirement can be artificially induced by trypsin treatment. It has been the custom of Singer's group to call the native enzyme "primer-dependent" even when the requirement or lack of requirement for primer has not been absolute in either case

(160). However, I think it safe to say that the primer requirement in both cases operates by a similar mechanism, and that their results are applicable to the general problem of primer requirement, especially since it is possible that the development of a primer requirement may be due to endogenous proteases (217).

Primers are certainly required for the polymerization of dNDP by PNPase (20), for GDP under normal reaction conditions (201), and possibly for the third class of inhibitors I mentioned previously (141). It is interesting to note that the stimulation of the polymerization increases with increasing chain length, while the strength of binding also increases (20,89,90). Thus oligonucleotides greater than 10-40 units long are more effective than shorter ones, and are more strongly bound. In numerical terms $(Ap)_2A$ is about 100 times less effective than $(Ap)_5A$. However, $(pA)_{50}$ can stimulate polymerization maximally at high concentrations but $(pA)_{1000}$ cannot completely overcome the lag phase at any concentration. This is explained as being due to the very slow rate of binding of polyA to the enzyme, although it is bound more strongly. The esterification of the oligonucleotide, and the nature of its constituent bases are also important. Thus, $(pA)_n$ oligonucleotides are an order of magnitude better than $(Ap)_{n-1}A$, and are an order of magnitude better than $(pU)_n$. I shall discuss the question of primer

requirement more fully under the heading of "Mechanism."

j) Mechanism

The mechanism of action of PNPase is not known exactly, although it is considered to be similar to that of other phosphorylases (26,115,172). Aside from its substrate specificity, however, the enzyme is unique among phosphorylases, in that it catalyzes cleavage and formation of P-O-P bonds rather than C-O-P bonds (103). In early studies, understanding of the processes of substrate binding and catalysis were hampered by the lack of well-defined pure substrates and analogues, and the correlation with structure was impossible without highly-purified enzyme preparations. Nevertheless, the early work outlined in broad terms the general characteristics of the reaction catalyzed, as I have discussed in the preceding sections. Recently groups at NIH, working with M. luteus PNPase, and at CNRS, working with E. coli, have made great advances in the fine details of the catalytic reaction. I shall attempt to confine myself to a discussion of the mechanical process involved, leaving the details of kinetics and physical structure for later sections.

In the polymerization reaction we have two basic steps; de novo synthesis and chain elongation. De novo synthesis, or chain initiation, can be described as the condensation of two NDP molecules to give a dinucleotide,

and chain elongation is the serial addition of NMP residues to this dinucleotide, or to an exogenous 3'OH oligonucleotide "primer." The elongation step presents no conceptual problems since it can be visualized as a binding of primer and NDP, with a subsequent displacement reaction whereby an NMP residue is joined to the 3' end of the primer, with liberation of inorganic phosphate. Since the strength of binding of oligonucleotides to the enzyme increases with chain length, it is not surprising that addition to short chain oligonucleotides is non-processive (131), that is, the oligonucleotide is detached from the enzyme after the addition of the new residue. With longer chains, which are much more strongly bound, the addition is processive, that is, successive additions occur without detachment of the growing chain. This process continues until the chain becomes so big that it is detached by mechanical or chemical means. This scheme favours the synthesis of very long polynucleotide chains, that is, the reaction is kinetically controlled, the likelihood of chain-elongation being higher than chain detachment. The reaction mixture eventually reaches the thermodynamically-favoured shorter chains, by the phosphorolysis of longer chains, until equilibrium is attained.

This reverse reaction of phosphorolysis is also processive (131,220). If only long-chain polynucleotide substrates are available, each chain is degraded to

"completion" before the resistant dinucleotide is released. With short-chain oligonucleotide substrates, the phosphorolysis is non-processive, with the whole population of substrates being sequentially degraded to the resistant dinucleotide. In this overall scheme the "exchange" reaction is considered to be simply an equilibrium between polymerization and phosphorolysis.

The sequences described above represent "the state of the art" as far as the mechanisms of PNPase action are concerned, and serve admirably in explaining the general characteristics observed for the chain-elongation and phosphorolysis activities. There remain, however, several puzzling areas, specifically concerning the chain-initiation, exchange and transnucleotidation activities.

In early work, with impure PNPase, it could always be speculated that the chain-initiation was due either to oligonucleotide contaminants, or to another enzyme. In the case of A. vinelandii PNPase, with its oligonucleotide "prosthetic group," it was suggested that this could serve as an endogenous primer (156). These views are no longer tenable, since pure E. coli (89) and M. luteus (160) PNPases have been shown to catalyze chain initiation in the absence of any contaminants. Besides being irreversible, the chain-initiation is slow in comparison with elongation (90). Further, with enzyme preparations which are highly primer-stimulated, the chain- initiation and elongation in unprimed

synthesis are processive from the very earliest stages, that is, the oligonucleotide is not released from the enzyme, as is the case with primed synthesis (160). These observations would suggest that de novo synthesis differs markedly from chain-elongation, either in mechanism or in the site of the catalytic steps, and there have been several proposals put forward to account for this, which are admitted to be inadequate (90).

Before detailing the generally accepted mechanism, I must point out several other discrepancies. Firstly, the exchange reaction is supposed to be an equilibrium polymerization-phosphorolysis, but there is no direct evidence that oligonucleotides undergo exchange at the 3' terminal residue, nor can PNPase cleave the α-phosphate. Exchange at the 5' phosphate end of the polynucleotide chain is impossible since PNPase has no phosphatase activity, nor can it degrade chains from the 5' end. Thus exchange is limited to 5' NDP as substrate, unless oligonucleotides are involved. Secondly, the product of phosphorolysis is a 5' mono-phosphate ended oligonucleotide (100). There is no evidence to suggest that any process involving de novo polymerization followed by phosphorolysis could yield a 5' di-phosphate ended oligonucleotide. Further, even if dinucleotides were not resistant to phosphorolysis, the 5' residue would still be a monophosphate. Thirdly, none of the proposed schemes take into account the transnucleotidation

activity. However, this activity has been shown only for the A. vinelandii PNPase (199) and until the present work, has never been confirmed, although all the review articles available take its existence into account. Finally, it is not possible that A. vinelandii PNPase uses its oligonucleotide "contaminant" as an endogenous primer for polymerization, since no PNPase has ever been shown to possess any endonucleolytic activity. Thus it is difficult to imagine the use of a specific endogenous primer for a physiologically insignificant activity such as exchange.

With these reservations in mind, the present theory (89,160) is that PNPase has multiple subsites, of which one class binds either oligonucleotide primer (or polynucleotide substrate in phosphorolysis) and another class which binds NDP substrate for the elongation (or the 3' terminal nucleotide in phosphorolysis). A subclass of the latter would be a site for the binding of NDP in de novo polymerization and this site would not be available for the reverse reaction of phosphorolysis of dinucleotides. Yet another site of a third class would be that for binding phosphate in the reversible polymerization-phosphorolysis. Finally there is another class (or subclass) of sites to account for the stimulation of polymerization by oligo- or polynucleotides (apart from the priming effect). This last type would, perhaps, accommodate 3' phosphate oligonucleotides, if they do in fact stimulate. Of course, there

must also be a site for binding of magnesium, if, as seems likely, it is bound in a step separate from the substrate (237).

The postulated sequence of events for de novo polymerization in this scheme is the binding of two NDP molecules, as such, to the enzyme at the appropriate subsites, and their condensation to form a dinucleotide. The elongation of this, or exogenous primer, continues by a similar mechanism with successive NDP molecules being bound to the enzyme, and then attached to the primer molecule. The chain-initiation site being separate from the primer site could account for the processive mode of unprimed synthesis as opposed to primed synthesis. Thus the reaction proceeds through an enzyme-NDP intermediate, by a "random-bi-bi" mechanism (160), that is, one in which each of the two substrates (NDP at one subsite and either primer or NDP at other subsites) are bound to their appropriate positions, without regard to whether or not anything is bound at the other positions. The great weight of evidence supports this hypothesis (20,103,45).

It is difficult, however, with this scheme, to account for the exchange activity and impossible to account for the transnucleotidation activity. Another possibility is that the reaction proceeds through an enzyme-NMP intermediate, that is, the incoming NDP is bound to the enzyme as NMP, with release of phosphate. A second NDP would then

also bind as NMP, and the two could be joined, etc. This scheme would account for all the discrepancies mentioned before, and could easily be incorporated into the one described in detail above. Unfortunately, all attempts to prove the existence of an enzyme-NMP intermediate have led to the opposite conclusion, that is, an enzyme-NDP intermediate, and in fact, kinetic data support the latter conclusion (103). This then is the present position with regard to the mechanism of PNPase action, and I will continue my treatment of it in the "Discussion" section, with reference to my own work.

k) Kinetics

It is beyond the scope of this work to study the detailed kinetic characteristics of the PNPase reaction, but a brief discussion is in order, if only to illuminate further the questions of mechanism, and the specificity of substrates. For a more complete discussion of the theory of kinetics I refer the reader to references 18, 20, 32, 42, 43, 44, 45, 46, 47, 89, 90, 103, 172 and 237, from which most of my discussion on mechanism and kinetics is drawn. I will not refer to the early work on PNPase, since the kinetic data, obtained at that time, although probably valid, has been superceded by recent, more detailed results. The early work has been adequately reviewed in references 80 and 83.

In the case of the polymerization reaction catalyzed by M. luteus PNPase, the V_{max} has been determined to be 3.0 μmol/min/mg (103), in optimal conditions, but increases with Mg^{++} concentration. The K_m also increases with the Mg^{++} concentration and is in the range of $3.1x10^{-5}M$ to $18.6x10^{-5}M$. The activation energy is 12.5 Kcal/mole. The K_m and V_{max} at first decrease with increasing salt concentration, then increase, accounting for salt activation and inhibition (9). A later paper, however, claims that no K_m or V_{max} could be calculated for M. luteus polymerization since the values depend not only on Mg^{++} and ADP concentration, but also on the concentration of an ADP-Mg^{++} complex (237). Further, the apparent ADP-K_m for the trypsin-treated enzyme is two orders of magnitude greater than for the native enzyme (160). The PNPases from A. vinelandii and E. coli are different from the M. luteus enzyme and their kinetic constants depend on the concentrations of "free" ADP and "free" Mg^{++}, and not in any way on the ADP-Mg^{++} complex concentration (237). In the case of A. vinelandii the ADP-K_m is $4x10^{-3}M$ and the Mg^{++}-K_m is $5x10^{-5}M$, while for E. coli the ADP-K_m is $2x10^{-3}M$ and the Mg^{++}-K_m is $5x10^{-5}M$. The activation energy for the E. coli enzyme is 14.6 Kcal/mole (90). Thus these enzymes are very similar to each other.

The bulk of the kinetic data in the literature refers to the phosphorolytic reaction, using either oligonucleotides or long chain polynucleotides as substrates.

For the M. luteus PNPase the V_{max} under optimal conditions is 0.52 μmol/min/mg (103), and the activation energy is 19.9 Kcal/mole. The K_m for polyA is around $3x10^{-4}M$, and for P_i around $6x10^{-4}M$. With increasing salt concentration the polyA-K_m first decreases, then increases while the V_{max} stays constant. Conversely, the P_i-K_m stays constant while the V_{max} increases. However, a more recent report has given the K_m for P_i as $1.8x10^{-3}M$ (203). The E. coli enzyme appears to bind polyA more strongly, since recent reports have given the K_m for polyA as $5x10^{-6}M$ (233) and $1.5x10^{-6}M$ (89), while the K_m for P_i is $7x10^{-4}M$ (90). E. coli mutant PNPases may be more similar to the M. luteus enzyme, since the E. coli K12 PNPase has a polyA-K_m of $1.5x10^{-5}M$, and the Q13 mutant $5.6x10^{-4}M$ (223). A further point is that for the normal E. coli PNPase, the polyA K_m increases with age (90), and may approach the value for M. luteus PNPase.

Detailed studies have been carried out on the phosphorolysis of well-characterized oligonucleotides. For M. luteus PNPase the K_m decreases with chain length, from $2.5x10^{-3}M$ for the trinucleotide, to $3.3x10^{-5}M$ for the octanucleotide (44). The V_{max} first increases then decreases, and remains constant after the heptanucleotide. Similarly with E. coli PNPase, the K_m for the trinucleotide is $2.2x10^{-4}M$, for the hexanucleotide $5.4x10^{-6}M$ (20), and for chain length 50, $7x10^{-7}M$ (89). The V_{max} decreases with increasing chain length up to 100 residues, then increases

slightly (89).

It is interesting that the K_m in phosphorolysis of a p(dA) terminal trinucleotide is 50% higher than for the all-ribose trinucleotide, while the V_{max} is ½ that of the all-ribose case (20). On the other hand, in the exchange reaction with $(pA)_2$ as stimulator, the stimulator-K_m for dADP exchange is 100 times that for ADP exchange, while the V_{max} in both cases is of similar magnitude. The elongation reaction is more complex. The oligo N-K_m for the addition of an ADP to $(pA)_2$ is $2x10^{-7}$M, and for the addition of dADP is $1.1x10^{-4}$M. The V_{max} in both cases is identical. For the addition to $(pA)_{40}$ the K_m drops to $1.8x10^{-5}$M but the V_{max} also drops to 1/18 that for $(pA)_2$. However, for the addition of dADP to $(pA)_2p(dA)$ the K_m is $4x10^{-4}$M and the V_{max} only 1/500 of that for $(pA)_2$.

To bring a little order into this formidable array of data, it appears that the A. vinelandii and E. coli enzymes are very similar, but differ from the M. luteus enzyme, at least for the polymerization reaction. They also differ quantitatively in the phosphorolysis reaction since E. coli PNPase binds a given oligonucleotide about ten times more strongly than does M. luteus PNPase. dADP is a poor substrate for PNPase in all the reactions, primarily because of the poor binding of dADP-containing substrates, as we might predict from the discussion in the "Inhibitors" section. In general, PNPase binds longer oligonucleotides

more strongly than shorter ones. Longer ones are better primers for this reason, up to a certain point, but they are phosphorylized less rapidly.

m) Physical properties

i) "Normal" PNPases

The topic of physical structures of various PNPases has recently been reviewed by Thang (215). In general, native PNPase from the usual sources is considered to be a single protein, catalyzing the polymerization, phosphorolysis and exchange reactions. The molecular weights have been determined as $2x10^5$ daltons for both E. coli (238) and A. vinelandii (214) PNPase, and $2.3x10^5$ daltons for M. luteus PNPase (127), measured by Sephadex G-200 gel-filtration and sucrose density-gradient ultracentrifugation and their electrophoretic properties compared (84). The A. vinelandii PNPase, of course, has an oligonucleotide prosthetic group, unlike the other two. On polyacrylamide gel electrophoresis, the A. vinelandii enzyme has been reported as single-banded (214) whereas E. coli and M. luteus (66,127) are multi-banded, suggesting the presence of isoenzymic forms. In the case of E. coli B, these multiple forms of PNPase can be attributed to the presence of an endogenous protease (216), and differ from the native protein, in that they are highly primer-stimulated, and very

sensitive to thermal inactivation. On extended proteolysis, the various forms become one form, which has been shown to have a molecular weight in the region of 1.6×10^5 daltons. Similar results are obtained with trypsin-treatment of native E. coli B PNPase. An interesting point is that there is a suggestion that the active form of E. coli PNPase may be phosphorylated in vivo (221), analogously to polysaccharide phosphorylase.

The trypsin-treated M. luteus PNPase has also been extensively studied, leading to new insights into the structure and mechanism of the native PNPase. Olmstead first observed that trypsin-treatment caused a preferential loss of CDP-polymerization activity (168,169,170). Early work dealt with the induction of primer-dependence by trypsin-treatment (130) and the concomitant change in the catalytic properties of the enzyme (65,170). Both single-banded and multi-banded preparations of the enzyme were transformed by trypsin-treatment into a single-banded species of higher electrophoretic mobility (67). The form obtained after trypsin-treatment is claimed to be the same as the native primer-requiring enzyme (127). The trypsin-treatment has been shown to induce primer dependence, which can be reversed by addition of β-mercaptoethanol (67,132). It appears that a certain free sulfhydryl group is necessary for the enzyme, either for a catalytic step, or for the maintenance of an active conformation. The trypsin-treated

or primer-requiring enzyme has a molecular weight of $2.2x10^5$ daltons, whereas the native enzyme is $2.6x10^5$ daltons (128), in contrast to earlier results. Treatment with guanidinium hydrochloride leads to a single species, M.W. $6.7x10^4$ daltons, in the case of the native enzyme, and $5.9x10^4$ daltons for the trypsin-treated enzyme. This suggests that M. luteus PNPase is a tetramer.

Quite different results have been published for the native E. coli B PNPase. The normal extract from E. coli B contains a PNPase with a M.W. of $1x10^5$ daltons, having only phosphorolytic activity for short-chain oligonucleotides (223). This was shown to be a subunit of the $2x10^5$ dalton PNPase. Further, SDS-polyacrylamide gel electrophoresis showed species of $3x10^4$ and $5.5x10^4$ daltons M.W. (215). Electron micrography of PNPase molecules suggested that the $2x10^5$ dalton native enzyme was composed of two trimers, i.e. six subunits, each of approximately $3x10^4$ daltons M.W. (229). The same authors observed that during polymerization the double-trimer becomes coated with nucleic acid, and this is reversible by addition of phosphate. If these results are factual, the conclusions are inescapable. A recent report by Lehrach et al. strongly suggests that the SDS-gel results, at least, are in error (137). The only subunit these authors could detect is the $9.5x10^4$ dalton subunit, and the supposed isoenzymic forms seen in normal gel electrophoresis cannot be detected in SDS-gels. The supposed $1.65x10^5$ dalton

partially-degraded PNPase differs from the $2x10^5$ dalton enzyme by no more than 30 amino acid residues or $3x10^3$ daltons. Thus, the question of the physical structure of E. coli PNPase must remain open for the present, although it quite definitely is at least dimeric.

The PNPase of Cl. perfringens is a special case. Early reports suggested that the purified enzyme lacks phosphorolytic activity and is specific for ADP polymerization (58) but it now is probable that the author had isolated only one of two forms of the enzyme (133). These forms are a heavy PNPase (M.W. $1.92x10^5$ daltons) and a light PNPase (M.W. $6.2x10^4$ daltons) (55). The heavy form is normally in higher concentration, and is similar to other PNPases in its physical and catalytic properties, except that ADP polymerization is highly stimulated by polybases (64). The light enzyme was shown to be a subunit of the heavy enzyme, and the two are interconvertible (96). Further, the light enzyme catalyzes only ADP polymerization, only with polylysine, and is absolutely dependent on β-mercaptoethanol, both for catalytic activity, and to prevent the reassociation of the subunits into the heavy form. Thus, the native enzyme is supposed to be a trimer. However, there is strong evidence that the appearance of the two forms is artefactual (69). Thang (215) has speculated that PNPases in general may be hexamers, the monomer having only phosphorolytic activity, but there is no firm

experimental evidence to support this hypothesis. The effect of trypsin-treatment of heavy Cl. perfringens PNPase has also been studied (69,70) and it was found that the trypsin-treated enzyme was no longer highly stimulated by polybases. It is interesting to note that polybases protected both Cl. perfringens and M. luteus PNPase from trypsin degradation, by an unknown mechanism.

ii) Other PNPases

Not all PNPases are like those described above. Altered PNPases exist in certain E. coli mutants which differ markedly from the normal E. coli PNPase, and the PNPases from such sources as H. cutirubrum and liver mitochondria, are quite unlike the normal bacterial PNPases.

Early reports suggested that E. coli Q13 (RNase I-deficient) lacked PNPase (17). However, it was suggested that the very high NDP-pyrophosphatase activity in this mutant masked the PNPase activity (162), and it was confirmed that an altered PNPase did exist (222). The mutant PNPase was found to have phosphorolytic activity but negligible polymerization activity in the standard assay, and that the M.W. of the mutant enzyme was around 10^5 daltons. Further, an altered 2×10^5 dalton PNPase was also found in very low concentration, and this was responsible for the polymerization activity. The "heavy" enzyme differs from that of E. coli B in that it is 100 times more active with

Mn^{++} in the polymerization reaction than with Mg^{++} (113), and the pH optimum is around pH 8 rather than pH 9. PolyA formation was stimulated appreciably by polylysine but not as much as for Cl. perfringens "heavy" PNPase. Phosphorolytic and exchange activities were negligible in all conditions due to a very high K_m for polyA (223). The "light" PNPase has no polymerization activity, phosphorolyzes only short-chain oligonucleotides and has a much higher Mg^{++} requirement than the normal $2x10^5$ dalton PNPase. It has been shown to be a subunit of the $2x10^5$ dalton Q13 enzyme (223). Similar results have been reported for E. coli mutants 1113B (36) Q_7, and Q_{27} (177,178).

H. cutirubrum PNPase is extremely interesting, because it appears to have one of the lowest reported molecular weights of any enzyme, from any source, e.g. 11,000 daltons (144). The enzyme is membrane-associated and is dependent on high ionic strength for both activity and stability (173). It catalyzes all three reactions typical of normal PNPases, but is dependent on Mn^{++} rather than Mg^{++} for all three, unlike any other PNPase.

Animal PNPases are also unlike the usual bacterial enzymes. The enzyme is particulate, firmly bound to the membranes and has only phosphorolytic activity (68,188,189, 190). The activating cation is Mg^{++}, although Mn^{++} can partially replace Mg^{++}, and the pH optimum is in the region of pH 8.6 to pH 9.2. The enzyme also appears to require

β-mercaptoethanol for stability and activity.

n) Metabolic role and uses of PNPase

As I have mentioned, PNPase is unlikely to have any synthetic role *in vivo* because of the lack of sequence-specificity in the products of the polymerization reaction. One cannot but discount the single report purporting to "prove" that PNPase has a synthetic role *in vivo* (16). It is probable that these authors have rediscovered the previously-observed phenomenon that PNPases from different sources differ in their affinity for polynucleotide precursors (63,166) and this is reflected in the composition but not base sequence of the synthetic polynucleotide. It cannot play a major role in sRNA metabolism, because of the resistance of this substrate to phosphorolysis (220). Ribosomal RNA (e.g. in yeast) is also resistant to PNPase action, because of the 3' terminal phosphates (79,157), but there are abundant phosphatases *in vivo* which could overcome this difficulty (116). PNPase has been implicated in the auto-degradation of ribosomes. The ribosomal PNPase of *Ps. aeruginosa* is active only in the 30S ribosomes (95), suggesting a control mechanism. Similarly, *E. coli* 30S ribosomes are readily degraded by PNPase (163) and the auto-degradation is inhibited by oligonucleotides (perhaps mRNA).

The general consensus is that PNPase is the primary enzyme involved in the degradation of messenger RNA, that is,

"rapidly labelled," single-stranded RNA. For instance, phage-induced RNA attached to ribosomes is selectively degraded by PNPase (49,184,191), and the same results have been reported for non-phage-infected cells (2,72). This postulated role of PNPase is attractive because specific control mechanisms exist; for instance, sRNA and related compounds inhibit the mRNA degradation (73). A most convincing result is the protection from degradation of polyU by ribosomes (37). The polyU is degraded only to the point of attachment to the ribosome, which would ensure that the "message" is read and immediately destroyed. Since PNPase phosphorolysis is processive, this would eliminate the possibility of "nonsense messages." Similar results have been shown for the mRNA of induced β-galactosidase (126).

However, this theory has been seriously questioned. Recent reports indicate that the "message" is read from the 5' end to the 3' end (158,159). Thus PNPase would destroy the message before it is read, unless the mRNA was protected from degradation. Further, there is direct evidence that in E. coli at least, the message is degraded from the 5' end by a hydrolytic pathway, that is, in the same direction as translation (41). In this case PNPase could play no major role in mRNA degradation. Nevertheless, B. subtilis mRNA is degraded from the 3' end by a phosphorolytic pathway, and PNPase is postulated to be the primary enzyme responsible

for this (59). In light of these results, it must be admitted that the physiological role of PNPase is, at present, unknown.

PNPase is a useful enzyme for the in vitro study of the synthesis and degradation of polyribonucleotides. In appropriate conditions, PNPase can be used to synthesize short-chain oligonucleotides of specific sequence (135,136, 211,212) which can be used to study the incorporation of amino acids into proteins (183). Of course, a great deal of knowledge concerning RNA structure has been derived from studies on well-defined polyribonucleotides, synthesized by PNPase (82,106,107,179,235). Finally, PNPase has been used in sequence determination of oligonucleotides, especially in the study of tRNA (148).

MATERIALS AND METHODS

I MATERIALS

Intermediates and enzymes were purchased from the following suppliers:

[^{14}C]NDP and [^{32}P]P_i-- Amersham Searle Corp., Don Mills, Ontario.

SDS (specially pure) -- B.D.H. (Canada) Ltd., Toronto, Ontario.

ApA and CpC -- Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, New York, U.S.A.

frozen A. vinelandii cells, oligonucleotides, polyA -- Miles Chemical Co., Elkhart, Indiana, U.S.A.

DEAE-Sephadex A-50, QAE-Sephadex A-50, Sephadex G-200 -- Pharmacia (Canada) Ltd., Montreal, Quebec.

unlabelled NDP -- P-L Biochemicals Inc., Milwaukee, Wisconsin, U.S.A.

BSA, hyaluronidase (bovine testis), pronase, tris, polylysine -- Sigma Chemical Co., St. Louis, Missouri, U.S.A.

DNase, RNase, lysozyme, crude snake venom, crystalline trypsin, soya-bean trypsin inhibitor -- Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.

PMB -- Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.

Cyanogum 41, TEMED -- E-C Apparatus Corp., Philadelphia, Pennsylvania, U.S.A.

Coomassie Brilliant Blue R250 was a gift from C.I.L., Montreal, Quebec.

Molecular weight marker proteins -- Schwarz/Mann, Picker Nuclear, Montreal, Quebec, Canada.

All other intermediates were purchased through Fisher Scientific Co., Ottawa, Ontario.

II METHODS

a) Measurement of enzymatic activity

i) Incorporation assay

The standard assay mixture contained, in a final volume of 0.1 ml, the reagents listed in Table 8. The assay is based on the method of Littauer and Kornberg (143). In general, the tris, Mg^{++}, ADP, and [^{14}C] ADP were stored frozen in a pre-mixed solution. This solution was thawed before use and 50 µl of it was used for each assay. Immediately after addition of the enzyme the mixture was incubated for 10 min at 37°, after which time the reaction was stopped with 0.1 ml of cold, 7% (w/v) perchloric acid. The mixture was allowed to stand in ice for at least 10 min; then 2 ml of cold 1% (w/v) perchloric acid were added. The precipitate was collected on a Whatman GF/C glass fibre filter with the aid of 2 ml cold 1% (w/v) perchloric acid.

Table 8

Constituents of standard ADP-incorporation assay

Reagent	µl/assay	Amount/assay	Concentration in assay
1.5 M tris-HCl buffer, pH 9.0	10	15 µmol	150 mM
0.1 M $MgCl_2$, pH 8	10	0.1 µmol	10 mM
8 mM EDTA, pH 8	5	0.04 µmol	0.4 mM
0.20 M ADP, pH 8	20	4.0 µmol	40 mM
[^{14}C] ADP	5	$3x10^4$-$6x10^4$ counts/5 min	-
PNPase	0-50	0-5 units	-
H_2O	to make 0.1 ml	-	-

The tubes were then washed with four 1 ml portions of the same perchloric acid, then the precipitate was washed in turn with 2 ml portions of 1% (w/v) perchloric acid and 50% (w/v) ethanol. The filters were placed precipitate-side up in metal planchettes, and dried for 10 min under a heat lamp. The radioactivity was counted in a Nuclear- Chicago low-background thin-window counter. Appropriate blanks were subtracted from the assay values.

One unit of incorporation activity is defined as the amount of enzyme catalyzing the incorporation of one μmol of [^{14}C] ADP into an acid-insoluble form in one hour. The specific activity of the ADP was estimated by spotting the appropriate amount of [^{14}C] ADP on a clean planchette, drying under a heat lamp and counting the radioactivity. The amount of ADP present in the [^{14}C] ADP was considered to be negligible in relation to the total amount of ADP present, and the radioactivity thus measured was taken to be that of the total ADP.

It is appropriate here to mention certain modifications of the standard assay procedure. In all assays of enzyme preparations having a specific activity greater than approximately 1,000 U/mg, or after the DEAE step described in the purification procedure, 10 μg of bovine serum albumin were routinely included in the assay. Other modifications include the addition of ApA and BME, and the amounts used are mentioned at the appropriate places in the "Results"

section.

The CDP-incorporation assay was carried out in the same way as the ADP-incorporation assay, except that CDP and [^{14}C] CDP were substituted for ADP and [^{14}C] ADP.

ii) Phosphorolysis assay

The standard assay mixture contained, in a final volume of 0.1 ml, the reagents listed in Table 9. The assay is based on the method of Grunberg-Manago et al. (88).

In general, the tris, Mg^{++} and EDTA were stored frozen as a pre-mixed solution. This solution was thawed before use and 25 µl of it were used for each assay, along with the polyA and [^{32}P] solutions, which were also stored frozen. The complete reaction mixture cannot be stored for more than a few hours because a precipitate forms in it. Further, storage of polyA at high pH, or in Mg^{++}-containing solutions, causes degradation of the polyA (70).

Immediately after addition of the enzyme the mixture was incubated at 37° for 10 min, after which time the reaction was stopped with 1 ml of cold 1% (w/v) acid-washed charcoal in 2.3% (w/v) perchloric acid. The suspension was allowed to stand in ice for at least 10 min with occasional mixing. The charcoal precipitate was collected on a Whatman GF/C glass fibre filter with the aid of 10 ml cold water, the test tube was rinsed with four 2.5 ml portions of cold water and the filter washed with 10 ml cold water. The

Table 9

Constituents of standard phosphorolysis assay

Reagent	µl/assay	amount/assay	Concentration in assay
1.0 M tris-HCl buffer, pH 8.2	10	10 µmol	100 mM
0.1 M $MgCl_2$, pH 8	5	0.5 µmol	5 mM
3 mM EDTA, pH 8	10	0.03 µmol	0.3 mM
polyA, 10 mg/ml	5	50 µg	0.5 mg/ml
50 mM $K_2H[^{32}P]O_4$	20	1 µmol $5x10^4$-$50x10^4$ counts/5 min	10 mM
PNPase	0-50	0-1 units	-
H_2O	to make 0.1 ml	-	-

filter was placed precipitate-side down in a metal planchette and dried under a heat lamp. Counting of radioactivity was as for the incorporation assay.

One unit of phosphorolytic activity is defined as the amount of enzyme catalyzing the incorporation of 1 µmol of [^{32}P] orthophosphate into a charcoal bound form in one hour. Note that some confusion exists in the literature as to the definition of a phosphorolysis unit--Thang (214), Singer and Guss (196). I have therefore recalculated the units quoted in the literature into the units defined above, where applicable. The measurement of the specific activity or [^{32}P] was analogous to that for [^{14}C] ADP, except that a charcoal-covered filter was placed on the dried residue prior to counting. The only modifications to this assay were the addition of 10 µg of bovine serum albumin at the same stages of purification as for the incorporation assay, and the addition of BME where appropriate.

iii) Exchange assay

The exchange activity of the enzyme was very rarely measured. On the one occasion when purification of the enzyme was monitored by the exchange assay, I used a modification of the procedure of Thang (214). The assay mixture contained, in a final volume of 0.1 ml, the reagents listed in Table 10.

The tris, Mg^{++}, EDTA and ADP were stored frozen in a

Table 10

Constituents of standard exchange assay

Reagent	µl/assay	amount/assay	Concentration in assay
1.0 M tris-HCl buffer, pH 8.0	10	10 µmol	100 mM
25 mM $MgCl_2$, pH 8	10	0.25 µmol	2.5 mM
1 mM EDTA, pH 8	5	0.005 µmol	0.05 mM
0.25 M ADP, pH 8	5	1.25 µmol	12.5 mM
0.1M $K_2H[^{32}P]O_4$	20	2 µmol $1x10^5$-$5x10^5$	20 mM
PNPase	0-50	0-1 phospholysis units	-
H_2O	to make 0.1 ml	-	-

pre-mixed solution, analogously to the other assays described. After addition of the enzyme, the procedure was exactly as described for the phosphorolysis assay. The amount of phosphate incorporated is given by the equation quoted in the "Introduction" section, and one unit is defined as the amount of enzyme catalyzing the incorporation of one μmol phosphate in one hour. Modifications to the procedure were analogous to those described for the incorporation assay.

iv) Transnucleotidation assay

No simple quantitative procedure has been developed for the measurement of transnucleotidation activity, and I did not attempt to devise one. The precise conditions used to demonstrate the existence of this activity are described under the appropriate figure in the "Results" section.

b) Other assays

i) Protein

Protein and nucleic acid were measured by the method of Warburg and Christian (234) using the data from reference 134. The absorbancies of the protein solutions were measured at 260 and 280 nm, whence one may calculate the protein and nucleic acid concentrations.

ii) RNA

When a more accurate estimate of RNA concentration was required, the method of Dische and Schwarz (56) was used, the procedure being as follows. Just before use, 120 mg of orcinol were dissolved in 2 ml 95% ethanol, then mixed with 28 ml $FeCl_3$ reagent (0.5 ml 10% w/v $FeCl_3.6\ H_2O$ in 100 ml conc. HCl). RNA-containing samples (0-40 µg) were made up to 1.0 ml with water and 2.0 ml of the orcinol-$FeCl_3$ reagent were added. The tubes were heated in boiling water for exactly 20 min, cooled to room temperature, and the absorbancies of the solutions were measured at 675 and 570 nm. The increase in the value $A_{675}-A_{570}$ is proportional to RNA concentration.

c) Electrophoretic procedures

i) Analytical polyacrylamide gel electrophoresis

The method is a modification of the method of Fitt et al. (67). The stock monomer solution contained per 100 ml 7 gm Cyanogum 41 and 130 µl N,N,N'N'-tetramethylethylenediamine (TEMED) in 0.2 M tris-borate buffer pH 9.0, 4mM EDTA. The solution was filtered and stored in the cold. To make 12 gels, 25 ml of this solution were mixed with 0.2 ml of freshly made ammonium persulfate solution (100 mg/ml in running buffer) and portions of the mixture were immediately

placed in vertically-mounted precision-bore glass tubing (0.5 cm i.d. x 8.0 cm), with a rubber stopper at the bottom end, and the tubes were tapped gently to dislodge air bubbles. Excess solution was removed so that all the tubes were filled to the same level (at a predetermined mark such that the gels after polymerization were 6 cm long), and 0.1 ml of running buffer were carefully layered on top of each solution. Polymerization generally occurred about 10 min after addition of the ammonium persulfate, and was indicated by an opalescence of the gel solution. After polymerization, the tops of the gels were washed with running buffer, the rubber stoppers were removed and the gel tubes were aligned in the apparatus (Buchler Polyanalyst). The top and bottom buffer compartments were filled with cold running buffer (0.05M tris-borate buffer pH 9.0, 1 mM EDTA) and the apparatus tapped to dislodge air bubbles. The apparatus was levelled so that the gels were vertical and the electrodes were connected (anode at bottom, cathode at top). The gels were pre-electrophoresed for 15 min at the running current. The samples (50-150 µl to which a few grains of sucrose had been added) were layered directly onto the tops of the gels. The samples were then "slow-loaded" by electrophoresing at 0.5 mA/gel for 15 min, after which time the current was raised to the running value of 1.5 mA/gel (approx. 60 V/12 gels) Electrophoresis was generally for 1-2 h. After electrophoresis the gels were carefully removed from their

tubes with the aid of a syringe, and placed in the appropriate staining solutions described below.

ii) Protein and nucleic acid stains

Protein bands were located in the gels by staining with Amido Black (naphthalene blue black). The gels were placed in 1% (w/v) Amido Black in 7% (v/v) acetic acid for 1 h, then destained overnight in running water. The method can detect as little as 0.5 µg protein.

Alternatively, protein bands were located by staining with Coomassie Brilliant Blue R250 (48). The gels were first fixed for ½ h in 10% (w/v) trichloroacetic acid, then stained for ½-1 h in 0.1% Coomassie Blue in 10% trichloroacetic acid. The protein bands can be viewed immediately, but become sharper after the gels have been allowed to stand overnight in 10% trichloroacetic acid. The gels cannot be stored in this solution for long periods of time because of serious shrinkage. The acid can, however, be leached out of the gels with several changes of water, without any deterioration of the bands. This method can detect less than 0.1 µg of protein but cannot be used for urea-containing gels, and even under normal circumstances may not be specific for proteins (see "Results" section).

Nucleic acids were located by staining with 0.2% (w/v) Methylene Blue in 0.4 M sodium acetate buffer, pH 4.7, (171), and the gels destained with running tap water. Care

must be taken not to destain for too long because the colour disappears if the destaining is carried too far, and fades even on storage.

iii) Localization of bands of enzymatic activity

PNPase can be located by carrying out an in situ ADP polymerization reaction and staining the polyA formed (67, 224). The gels are incubated at 37° for 1-2 h in a mixture containing 0.15 ml 0.20 M ADP, 0.20 ml tris- Mg^{++}-EDTA (in the proportions described for the ADP-incorporation assay), 0.02 ml ApA (20 mg/ml and H_2O (sufficient to cover the gel in the test tube). The product in the gels was stained for 2 h in 1% (w/v) Acridine Orange, 2% (w/v) $LaNO_3$, in 15% (v/v) acetic acid, and the background colour was destained overnight in running tap water. The method as described can detect 0.1 ADP-incorporation units, or less if longer incubation times and more concentrated reagent solutions are used.

iv) SDS gel electrophoresis

An estimate of the molecular weight of a protein can be made using a modification of the method of Dunker and Rueckert (60). The monomer solution contained 5% (w/v) Cyanogum in 0.1 M phosphate buffer pH 7.2, 0.1% (w/v) sodium dodecyl sulfate, 0.1% (v/v) TEMED. Details of the preparation

of the gels were as described above for the normal gels. There was no pre-run, and the actual run was carried out for approx. 4 h at 150 V, using 0.1M phosphate buffer pH 7.2 at room temperature as electrode buffer. The protein samples were prepared by incubating the protein for ½-1 h at 45° in 4M urea, 1% (w/v) sodium dodecyl sulfate, 1% (v/v) BME. Aprox. 1-10 µg of protein in 20-50 µl solution were layered onto each gel. In these conditions the rate of migration of a protein is proportional to its molecular weight, regardless of other factors. M.W. markers were ovalbumin (45,000), albumin (67,000), gamma globulin (160,000).

v) Preparative-scale polyacrylamide gel electrophoresis

The method is a scaled-up version of the analytical gel procedure, using a 7% gel in 0.2 M tris-borate buffer pH 9.0, 4 mM EDTA. The running buffers contained 0.05 M tris-borate buffer, pH 9.0, 1 mM EDTA, and the same buffer was used for elution. The apparatus used was the Shandon Preparative Acrylamide Electrophoresis Apparatus, and the preparation of the gel, and operation of the equipment was according to the manufacturer's instructions, except that no stacking or sample gels were used. The annular running gel (above the elution chamber) was measured after the experiment, and found to be 3.5 cm long and approximately 3 mm across the annulus. After preparation of the gel and assembly of the apparatus, the equipment was placed in a

cold room, levelled, and cold water was passed through the outer-jacket and inner "cold-finger." The flow of buffer through the elution chamber was started and maintained at 5 ml/h for the duration of the experiment. After an initial pre-run at 200 V (approx. 14 mA) the enzyme sample, containing 10% sucrose, was layered on to the surface of the gel under the upper electrode buffer. The enzyme was slow-loaded at 100 V for 1½ h and electrophoresed at 200 V for 24 h. 3 ml samples were collected and assayed for protein and enzyme activity. The only modification to this procedure was the addition of 10 mM GSH to the enzyme sample, in an experiment carried out by Mrs. G. Zahror de Behrens, to try and improve the recovery of activity (176).

vi) Isoelectric focusing

Attempts were made to purify the enzyme, and to determine the pI of the enzyme by "isoelectric focusing." The apparatus used was the LKB 8100-10 (110 ml) Electrofocusing Column, using LKB Ampholine carriers, either pH 3-10 (LKB 8141) for preliminary experiments, or pH 3-6 (LKB 8142) for later experiments. The apparatus was operated according to manufacturer's instructions. In the preliminary experiments, the pH gradient was stabilized by a discontinuous sucrose gradient set up according to the protocol given by the manufacturer. In this case the enzyme sample (before electrofocusing) was mixed into several of the

central layers of the gradient. In later work I used a continuous sucrose gradient, with the enzyme distributed throughout. The current was turned on and the voltage raised from 300 V to 1,000 V over several hours so that the power (VxA) never exceeded 0.5 W. Electrofusing was continued until equilibrium (zero current) was attained, after approximately 48 h. The power supply was then disconnected and the Ampholine solution was run out of the column. 2 ml fractions were collected and measured immediately for enzyme activity, pH, and absorbancy at 280 nm.

d) Preparation of enzyme

The following description is of a typical preparation of highly purified Azotobacter vinelandii PNPase, in its reduced form. The standard buffer used was 10 mM tris-HCl pH 8.2, 1 mM EDTA, 1 mM BME (TEB 8.2). This latter notation is used throughout this work to describe the buffers used. For instance 10xTEB 8.2 refers to 100 mM tris-HCl pH 8.2, 10 mM EDTA, 10 mM BME, and TE 7.4 refers to 10 mM tris-HCl pH 7.4, 1 mM EDTA, et seq. All operations were carried out in the cold-room (0-5°) unless otherwise mentioned.

i) Crude extract

100 gm of A. vinelandii frozen wet cell paste were placed in 300 ml 10xTEB 8.2 and stirred until the mixture

was homogeneous (about 1 h). The cells were disrupted ultrasonically in a Bronwill Biosonik, at maximum intensity for 15 min. The mixture was centrifuged ½ h at 38,000 x g and the supernatant decanted. The precipitate was resuspended in another 300 ml of 10xTEB 8.2 and the same procedure repeated. The combined supernatants from the two centrifugations comprised the crude extract.

ii) Ammonium sulfate fractionation

The crude extract was immediately adjusted to 25% saturation in ammonium sulfate (0.144 g/ml crude extract) (78), stirred ½ h and centrifuged at 28,000 x g for ½ h. The precipitate was discarded and the supernatant adjusted to 60% saturation in ammonium sulfate (0.230 g/ml of 25% saturated solution), stirred for ½ h and centrifuged for ½ h as before. The supernatant was discarded and the precipitate dissolved in a minimum volume (approx. 200 ml) of 10xTEB 8.2. The solution was dialyzed, once for 4-5 h against 4 l distilled water, then once overnight against TEB 8.2. The dialyzed solution was the AS fraction.

iii) DEAE-Sephadex I

The ion exchanger was prepared by suspending DEAE-Sephadex A-50 powder in distilled water and washing by decantation until the supernatant was clear. The suspension was adjusted to 0.1 M NaCl in TEB 8.2 and washed several

times with this buffer until the pH of the supernatant reached the nominal value. A column (2.5 x 90 cm) of the exchanger was poured as a 50% slurry, the bed was allowed to settle, and the column was then washed with at least one column volume of 0.1 M NaCl in TEB 8.2, at a flow rate of 20 ml/h.

The AS fraction was adjusted to 0.1 M NaCl with 1 M NaCl and adsorbed on the column. The column was washed with one column volume of 0.1 M NaCl in TEB 8.2. The enzyme was eluted with a 1.5 l concave gradient, 0.25-0.50 M in NaCl in TEB 8.2 (formed in a Buchler Varigrad gradient maker, 500 ml each of 0.25 M NaCl in the first two chambers, 500 ml of 0.50 M NaCl in TEB 8.2 in the third chamber). The eluted fraction containing the enzymatic activity was adjusted to 60% saturation in ammonium sulfate, centrifuged, and the precipitate redissolved in a minimum volume of TEB 8.2. The concentrated active fraction (8-15 ml) was the DEAE I fraction.

iv) G-200

The DEAE I pool fraction was applied directly onto a column of Sephadex G-200 (2.5 x 90 cm), pre-equilibrated with TEB 8.2 and the enzyme eluted at 8-10 ml/h with the same buffer. The enzymatically active fractions were pooled to give the G-200 fraction.

v) DEAE-Sephadex II

The G-200 pool was adjusted to 0.25 M NaCl and adsorbed on a column (1.5 x 20 cm) of DEAE-Sephadex A-50 prepared as described above, but pre-equilibrated with 0.25 M NaCl in TEB 8.2. The column was then washed with at least one column volume of this buffer and the enzyme eluted with a 1.5 l concave gradient 0.25-0.40 M NaCl in TEB 8.2, at a flow rate of 20 ml/h. The enzymatically active fractions were pooled and dialyzed against TEB 8.2. The dialyzed solution was the DEAE II fraction.

vi) QAE-Sephadex

The DEAE II pool was adjusted to 0.26 M NaCl and adsorbed on a column (1.5 x 10 cm) of QAE-Sephadex A-50 prepared in a manner similar to the DEAE columns. The column was washed with one column volume of 0.26 M NaCl in TEB 8.2 and the enzyme eluted with a 1 l linear gradient, 0.26-0.34 M NaCl in TEB 8.2. The enzymatically active fractions were pooled, dialyzed against TEB 8.2 and concentrated by suction to approx. 10 ml in a Sartorius collodion-bag concentration apparatus. The concentrated fraction was the QAE pool and was the enzyme preparation on which most of the work in this thesis was done.

RESULTS

a) Purification

Table 11 shows the results of a typical preparation of A. vinelandii PNPase, starting from 125 g wet weight of bacteria. Usually over 80% of the units are released during the first sonic disruption step, so I think it safe to say that the procedure efficiently extracts most of the PNPase present in the bacteria. In my experience, shorter periods of sonication have given an enzyme of slightly higher specific activity, but in somewhat lower yield. I should perhaps emphasize that in the early stages of purification up to the DEAE II step, I was interested mainly in maximum yield of enzyme, rather than in maximum purification. Temperature control does not appear to be critical during the preparation of the crude extract, since on occasion it rose as high as 20° during the sonication, with no adverse effects on the yield or stability of the enzyme.

The ammonium sulfate fractionation as shown here gives only a two-fold purification. I have been able to obtain three-fold purifications by retaining the fraction precipitating between 30 and 50% saturation but with only 80% of the units in the crude extract. Although the purification is not spectacular in view of the results from the

TABLE 11

Typical purification of PNPase in TEB 8.2 buffer.
Preparation from 125 g bacteria

Enzyme fraction	Total protein (mg)	ADP-incorp. activity (units)	Specific activity (units/mg)
crude extract	24,100	75,800	3.2
ammonium sulf. 25-60%	12,800	73,200	5.7
DEAE-Sephadex I	560	94,000	170
G-200	230	94,200	410
DEAE-Sephadex II	34	87,500	2600
QAE-Sephadex	7.1	45,600	6400

ion-exchange columns, this step was found to be essential to the success of the method. The ammonium sulfate fractionation and/or the subsequent dialysis remove unspecified substances which interfere with the adsorption of the enzyme to DEAE-Sephadex.

The enzyme at this stage of purification is strongly adsorbed to DEAE-Sephadex in 0.1 M NaCl in TEB 8.2 but in the presence of 0.2 M NaCl, most of the enzymatic activity is not adsorbed, and passes through the column with no purification. However the enzyme, once adsorbed, stays bound to a concentration of 0.28-0.32 M Cl^- ions, as measured by the method of Schales and Schales (186). The enzyme emerges from the column as a sharp symmetrical peak when eluted with a long shallow or concave salt gradient. If one attempts to elute the enzyme with buffers of fixed concentration (batchwise elution), the enzyme is eluted with very poor purification and in very low yield. In early work on the A. vinelandii PNPase, I attempted to purify the enzyme by DEAE-cellulose chromatography, but it appeared to be unstable even for periods of 6 h on this material. PNPase is stable for periods of at least one week while adsorbed to DEAE-Sephadex, and in fact I usually recover more units of enzymatic activity from these columns than are present in the AS fraction. I suspect this to be due to the removal of inhibitory substances during the purification. Although the activity of the enzyme

preparation is somewhat unstable until adsorbed to the DEAE-I column, it is quite stable throughout the succeeding purification steps.

The G-200 step has proved to be the most variable in the purification, solely due to technical problems. To obtain adequate separations, the enzyme must be applied in a volume of less than 15 ml, i.e. less than 3% of the column volume, which means that the DEAE II fraction must be concentrated to approx. 50 mg/ml protein concentration. This is most easily done by precipitation with ammonium sulfate, but it is sometimes difficult to redissolve the precipitate completely, and any undissolved particulate matter can clog the column. One must pay careful attention to the hydrostatic pressure and flow rate of the column. Optimum purifications are obtained at zero hydrostatic pressure and a flow rate of 1.5-2.0 ml/cm^2 column cross-section/hr. The enzyme emerges at a volume equal to the void volume plus approx. 30%, which indicates a molecular weight in the region of 2×10^5 daltons. I have obtained purifications of up to five-fold during this step and it is probable that its reliability could be greatly improved by using the upward flow technique for which the necessary equipment was unavailable in the laboratory at that time.

The DEAE II step is carried out similarly to the DEAE I step, except that the enzyme is adsorbed in 0.25 M NaCl, and is eluted with a shallower gradient. I have on

occasion been able to obtain an enzyme of at least 95% purity after this step, based on gel electrophoresis. In general the enzyme is only about 50% pure, and an additional purification step must be carried out. The reason for this appears to be the variability of the G-200 step.

The final QAE step is very simple, and invariably yields an enzyme of very high purity. Only the fractions of highest specific enzymatic activity are pooled, which accounts for the apparent poor yield. Further, some activity is lost during the concentration step in the Sartorius collodion-bag apparatus, probably due to denaturation at the air-liquid interface, a problem that could be avoided with more sophisticated ultrafiltration equipment now available.

I am convinced that the success of my method is primarily due to the maintenance of the enzyme in the reduced state at all times. For example, in Table 12, if one measured the activity without BME, one would consider this to be a failed preparation, but assaying with BME shows that it is a rather good one. I feel that the poor yields and reproducibility of earlier published procedures were caused by just such an effect. Further, I observed that in general, purifications were worse in the absence of BME, because the oxidized enzyme was eluted in broad peaks from ion-exchange columns, and addition of BME to all the buffers greatly improved the resolution. I shall treat the

TABLE 12

Oxidation of PNPase during preparation in tris 7.4 buffer.
Preparation from 75 g bacteria

Enzyme fraction	Total protein (mg)	without BME		with BME	
		ADP-inc. (units)	Specific activity (units/mg)	ADP-inc. (units)	Specific activity (units/mg)
crude extract	6,300	20,000	3.2	20,000	3.2
ammonium sulf. 25-55%	3,900	20,700	5.3	21,700	5.6
DEAE-Sephadex	170	16,400	91	29,000	170
G-200	51	8,100	160	26,000	510

reversible oxidation of A. vinelandii PNPase in detail in a later section.

The purification varies somewhat with the pH of the buffers employed. The enzyme can be purified to a great extent (95%) at pH 7.4 (with BME), in a minimum number of steps (Table 13), but an extra step is needed to attain acceptable purity. Purification at pH 8.2 is somewhat poorer at any given stage (compare Tables 11 and 13). However, as I shall demonstrate later, the enzyme is much more stable at this pH than at pH 7.4, and I therefore chose pH 8.2 for all succeeding preparations. Purifications at pH 9.0 were no better than those at pH 8.2. Further, most published purification procedures for PNPase were performed at pH 8.2. For this same reason, I included EDTA in all my buffers, although purifications in its presence or absence were identical.

My best preparation, both in terms of overall yield of units and highest specific activity, is shown in Table 14. This points out a puzzling discrepancy which can be noticed by comparison with the other tables. Although the final specific activity is 8,800 units/mg, the enzyme was no purer, judging by gel electrophoresis, than that shown in Table 11, whose final specific activity is only 6,400 units/mg. Further, the specific activity after the second DEAE-Sephadex column is 7,300 units/mg, yet this enzyme was of purity equivalent to that shown in Table 13, both of

TABLE 13

Purification of PNPase in TB 7.4 buffer.
Preparation from 75 g bacteria

Enzyme fraction	Total protein (mg)	ADP-incorp. activity (units)	Specific activity (units/mg)
crude extract	8700	39,000	4.5
Ammonium sulf. 25-55%	4400	40,000	9.1
DEAE-Sephadex I	190	42,000	220
G-200	40	24,000	600
DEAE-Sephadex II	5.6	22,000	4000

TABLE 14

Best purification of PNPase in TEB 8.2 buffer.
Preparation from 125 g bacteria

Enzyme fraction	Total protein (mg)	ADP-incorp. activity (units)	Specific activity (units/mg)
crude extract	21,000	62,000	3.0
ammonium sulf. 25-60%	11,000	62,000	5.8
DEAE-Sephadex I	264	69,000	260
G-200	40	50,000	1300
DEAE-Sephadex II	7.9	60,000	7300
QAE-Sephadex	4.7	41,200	8800

which I judged to be no less than 95% pure by integrating densitometry of polyacrylamide gels.

There are three generally employed techniques for measuring protein concentrations in dilute solutions: the micro-biuret reaction (242), the Lowry procedure (145), and the Warburg and Christian technique (234). I found that all three were affected by varying concentrations of salt and BME, such as found in my enzyme fractions, but the first two had the added serious disadvantage of not being directly proportional to protein concentration in these conditions. The Warburg and Christian technique <u>was</u> proportional, and had the advantage of being very fast, therefore I chose it as my standard assay for protein. I must stress that all of these methods measure protein concentration only in relation to a given reference protein, and it is quite likely that PNPase is unlike yeast enolase, the standard for the Warburg and Christian technique. Thus, specific activities (enzyme units/mg protein) are not rigorous criteria for establishing purity, not even for estimating relative purifications. I therefore do not attach any particular significance to variations in specific activity, and do not draw any conclusions based on estimates of protein concentration.

b) <u>Stability</u>

The stability of the pure enzyme varies greatly with pH. For example, the preparation shown in Table 13, which

was 95% pure, was quite unstable at pH 7.4. After one day it had only 68%, after 3 days 31%, and after 1½ weeks 3% of the final activity shown in the table. None of this activity could be restored by ApA or BME in the assay. Another similar preparation lost 30% of its activity in one week when prepared and stored at pH 7.4.

Table 15 shows the stability of a 95% pure enzyme as a function of pH. The enzyme was mixed with tris buffer of the indicated pH and assayed immediately with ApA. Note that the high concentrations employed affected the activity in the assay, thus, the base value for pH 5.0, as one example, is only 50% of the value in the standard assay at pH 9.0. The base values for each pH are, therefore, those determined in each individual set of conditions. It can be seen that the stability of PNPase decreased as the pH was lowered, although at pH 7.4 this effect was partially alleviated by an increase in ionic strength.

It is interesting that during the seven weeks of the experiment, the enzyme developed a primer requirement: in every case the final stimulation by ApA was roughly 200%. The losses shown in the table are in addition to any increase in primer requirement, and were not due to oxidation, since the enzyme was prepared and stored in 10 mM BME. Because of these results, all succeeding preparations were done in TEB 8.2 buffer.

There are, of course, other factors which affect the

TABLE 15

Stability of ADP-incorporation activity as a function of pH

pH	Tris mmol/l	NaCl mmol/l	Time	
			3 weeks	7 weeks
			residual activity	
5.0	500	-	0.48	0.19
7.4	0.5	-	0.60	0.19
7.4	0.5	500	0.78	0.31
7.4	500	-	0.62	0.34
8.2	500	-	0.92	0.59
9.0	500	-	0.96	0.63

instability of the enzyme. To mention briefly a few: 25% glycerol or 0.5 M BME at pH 7.4 irreversibly destroy the enzymatic activity within 3 weeks; BSA (0.5 mg/ml) has no effect, while ApA (5 mg/ml) is equivalent to 0.5 M NaCl in its stabilizing effect.

Aside from the effect of aging, which I will duscuss later, the pure enzyme in TEB 8.2 was quite stable for appreciable periods of time when kept in the refrigerator, losing only 15% of its activity, measured in the presence of ApA, during a five-week period, and in fact, suffered no loss of activity even after incubation at 37° for 18 h. However, after freezing overnight and thawing, the enzyme at pH 8.2 lost 23% of its activity and at pH 7.4, 95%.

c) Purity and electrophoretic properties

During the course of most of the studies embodied in this thesis, one of the most powerful analytical tools I employed was polyacrylamide gel electrophoresis. In fact, my claim that I have obtained a PNPase preparation from A. vinelandii, containing only one protein species, is based on the use of this method in nine different combinations of gel concentration and pH. The enzyme obtained immediately after the final QAE step (Table 14) exhibited only one band in the gels for both protein and activity, with one serious reservation: when staining the protein gels with Coomassie Blue, a very thin, intense band with one-half the mobility

of the major band appears. I call this the "anomalous" band. By integrating densitometry of the Coomassie Blue stained gels, the band has only 3-5% of the absorbance of the enzyme band, which would suggest that the impurity constitutes less than 5% of the preparation, assuming that the colorimetric properties of Coomassie Blue follow the Beer-Lambert law. The "anomalous" band is not normally revealed by staining with Amido Black or Methylene Blue stains, but if the gel is drastically overloaded with the enzyme preparation (40 µg) the "anomalous" band appears very faintly. Since I have found that the limit of detectibility with Amido Black is of the order of 0.5 µg, this suggests that the "anomalous" band represents approximately 1% of the preparation. It is not possible to measure this densitometrically, since I have found that the Amido Black stain is only proportional to protein concentration below 10 µg protein/band, nor does the "anomalous" band appear in the densitometric tracings.

The "anomalous" band does not stain with Acridine Orange, whereas PNPase does, so it does not appear to be RNA, nor does it have any enzymatic activity. When the enzyme preparation is treated with pronase, DNase, hyaluronidase, α-amylase, or crude snake venom, the "anomalous" band remains unchanged. These experiments were carried out in a standard set of conditions, that is, the lytic enzyme was dissolved in TEB 8.2 buffer and added to the PNPase

preparation prior to incubation at 37°, but in every case, the treatment altered the electrophoretic pattern of PNPase in some way, so the lytic enzymes had activity in these conditions.

Although the "anomalous" band can not be separated from PNPase by the usual column procedures, it can be separated by preparative scale polyacrylamide gel electrophoresis. I was able to obtain a PNPase which had only one band on the analytical gels, but with only 5% yield. Mrs. G. Zahror de Behrens, who continued some of my work, managed to improve this to 18% yield, but this does not appear to be a promising preparative procedure. She was able to isolate the pure "anomalous" component as well, and confirmed that it was unaffected by the lytic enzymes mentioned above, nor by lysozyme, so its nature remains a mystery.

There is no correspondence between the activity band and any specific protein band until after the second DEAE-Sephadex column. At this point, of course, the enzyme protein constitutes the major part of the protein in the preparation. The enzyme was tested for the presence of nucleases, which might interfere with ADP-incorporation assays, by incubating it with polyA and $MgCl_2$ in the absence of phosphate, and examining the supernatant after uranyl acetate-perchloric acid treatment for 260 nm absorbing material. This was done at pH 7.0, 8.0 and 9.0 and in each case there was no increase in absorbancy with time.

PNPase itself is closely associated with nucleic acid. The enzyme band on polyacrylamide gels could be detected by staining with Acridine Orange or Methylene Blue without prior incubation with substrates, provided sufficient protein was applied to the gel. In general, the QAE enzyme has an A_{280}/A_{260} ratio around 0.75, which corresponds to approximately 7% (w/w) of RNA. This was confirmed directly by the orcinol reaction of Dische and Schwartz (56). Even after the preparative gel electrophoresis step, the absorbancy ratio was 0.83, i.e. 6% (w/w) nucleic acid, and on occasion this value was attained after the QAE step. The orcinol reaction showed that this last preparation contained 16 µg of RNA in 210 µg of protein, measured by the method of Warburg and Christian. This nucleic acid cannot be separated from the enzyme by any physical means, although it could be destroyed by RNase digestion. Gel electrophoresis of RNase digested samples of PNPase, showed that the enzyme-associated RNA was no longer present, and that the enzyme protein band had a higher mobility than the undigested control enzyme.

The PNPase was inactivated by this treatment (Fig. 1). In this experiment RNase at the appropriate concentration was added to an equal volume of a PNPase preparation (0.08 mg/ml, spec. act. 9,000 units/mg) to give the indicated ratio of PNPase to RNase. This was then incubated at 37° for 10 min, portions were withdrawn and

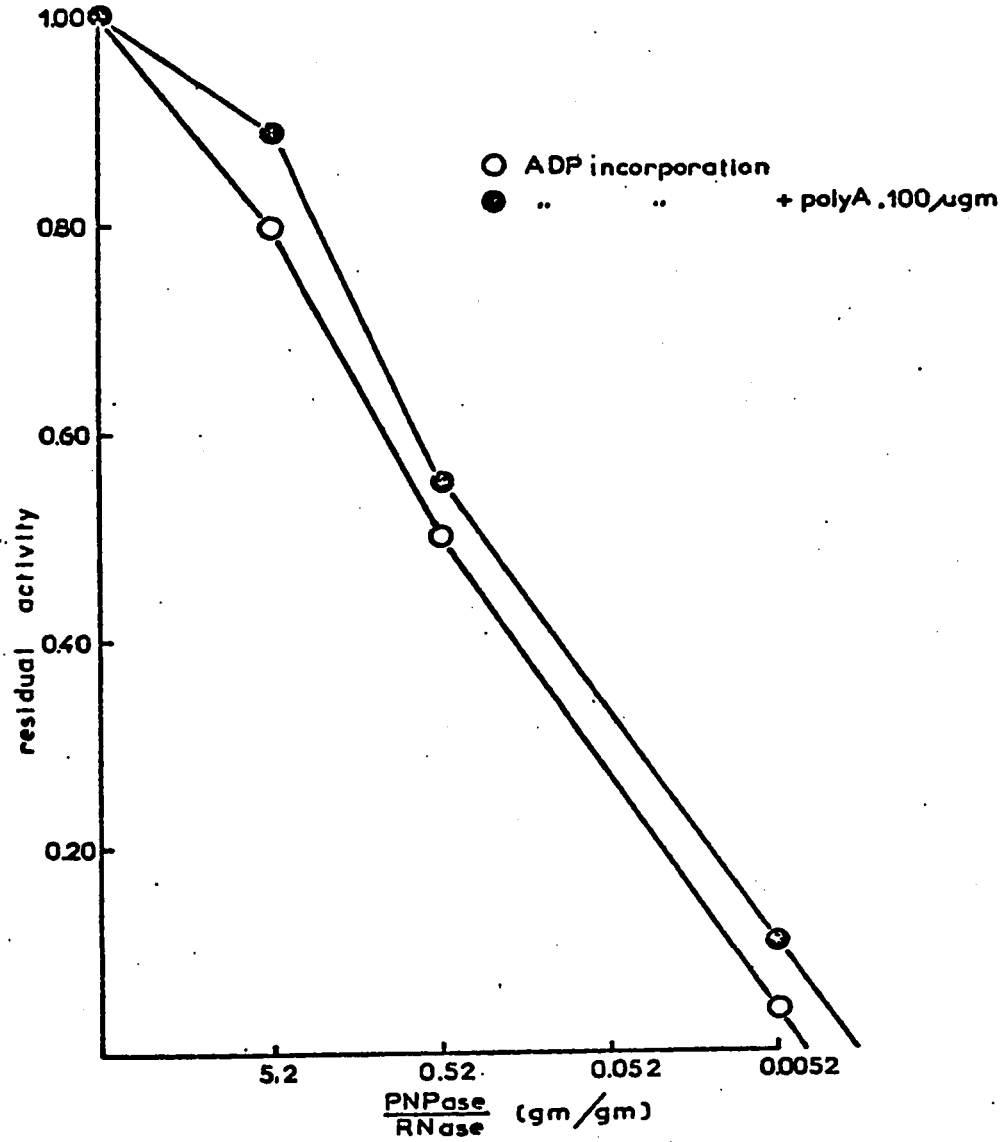


FIG. 1. Effect of RNase A digestion

assayed for ADP-incorporation activity a further 10 minutes. The PNPase concentration in the incubation was 3.1 μg/ml. It is obvious from the graph that the activity of PNPase is very sensitive to the action of RNase. PolyA (100 μg in the assay) does not appreciably reverse the effect and ApA (100 μg) is only about one-half as effective as polyA in this respect. Although it is not shown here, it appears that the phosphorolytic activity is slightly more sensitive to RNase than is the ADP-incorporation activity. In another experiment I found that if the first incubation was carried out at ice temperature, the PNPase retained 95% of its incorporation activity (with ApA) when the PNPase/RNase ratio was 0.52, while at 37° it retained only 60% of the activity. In the same experiment the enzyme retained 58% of its phosphorolytic activity at ice temperature and 42% at 37°. I did not have an inhibitor for RNase similar to the soybean inhibitor for trypsin. For example, I found that Bentonite, in concentrations that inhibit RNase, was also quite effective in inhibiting PNPase irreversibly. I should mention that the inactivation is not due to degradation of polyA by RNase (13), since this occurs only in conditions of high ionic strength, quite unlike my conditions. Further, in my conditions, RNase would not be expected to assume the form active in polyA degradation (53).

To return to the question of purity, I found that the freshly prepared, reduced PNPase migrated as a single

band in gel electrophoretic experiments in a variety of conditions. The QAE enzyme was subjected to electrophoresis in 5%, 7%, and 10% (w/v) gels, in each case at three different pHs (7.4, 8.2 and 9.0). The procedure was as described in the "Methods" section except that for the different gel concentrations Cyanogum 41 was used at the appropriate concentration (with the Cyanogum : TEMED ratio kept constant), and for the different pHs the appropriate tris-borate buffer was used. (C.f. standard gel run, of course, is in a 7% gel at pH 9.0.) In every case there was only a single protein band in the gel.

In an attempt to purify the enzyme further, I subjected it to iso-electric focusing in the LKB-ampholine apparatus (see "Methods"). In early attempts with 95% pure PNPase, the enzyme precipitated heavily in the column and the pI was determined to be around pH 4.6. Later with the pure enzyme, I used much smaller amounts and found the pI to be 4.1 ± 0.2. This is an average value of several runs, one of which is shown in Figure 2. The enzyme could be detected in the column by a faint opalescence. The possibility therefore remains that the value obtained represents only an upper value for the pI. The method could not be used for purification, since considerable losses of activity occurred during electrofocusing (max. 20% recovery of activity), probably as a result of prolonged exposure (48 h) of the enzyme to pHs below 8.2. The mixed ampholine reagent

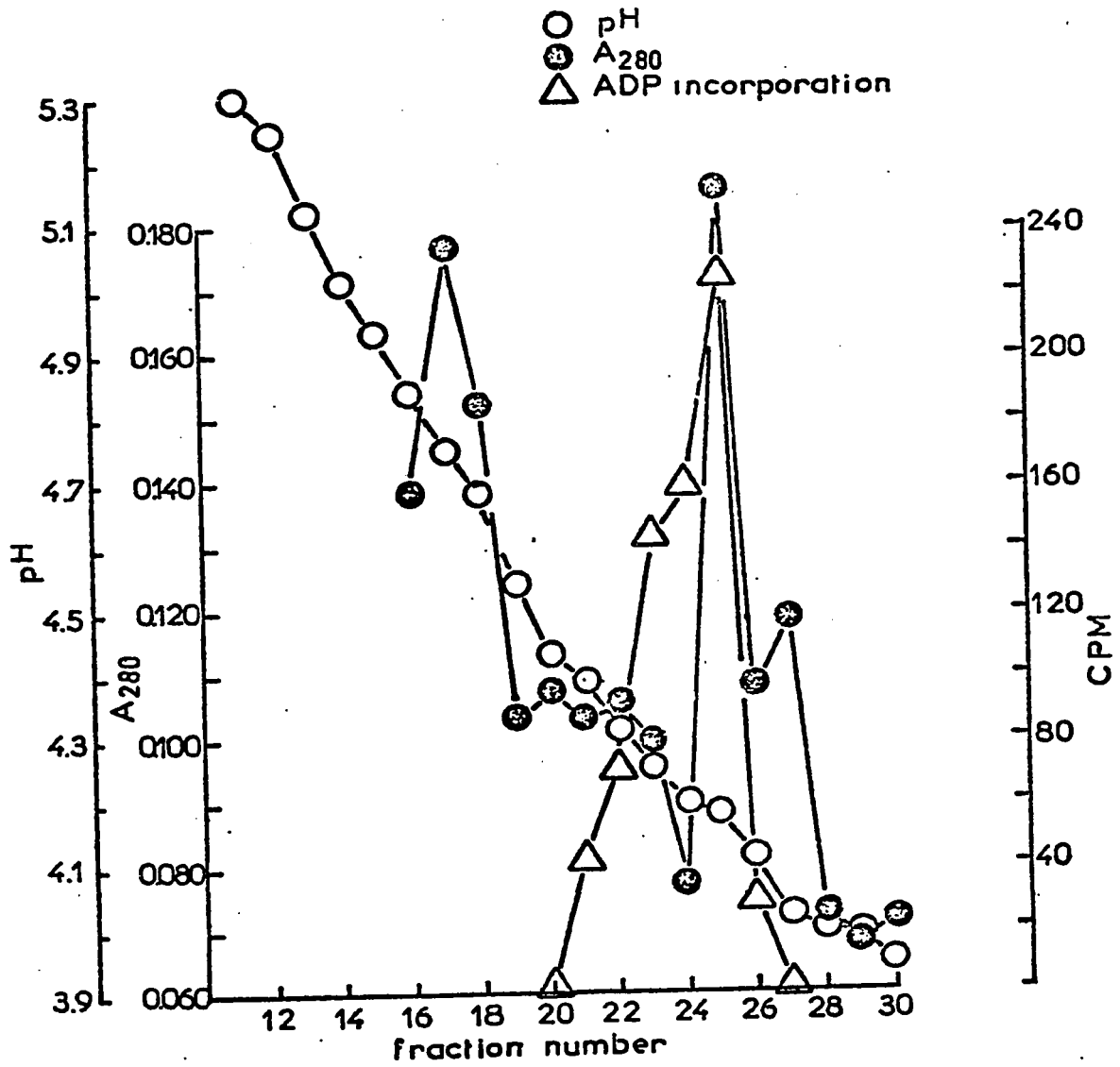


FIG. 2. Results of isoelectric focusing

(pH 3 - 10) itself has no significant effect on the enzyme assay. The peak enzyme fraction after electrofocusing has electrophoretic properties identical to those of the QAE enzyme; it is still closely associated with nucleic acid and the "anomalous" band is still present, so the pI determined must represent that of the enzyme-nucleic acid-"anomalous" component complex.

More interesting are the results of SDS gel electrophoresis of PNPase (see "Methods" section). This technique involves pre-treatment of the protein of interest with urea, BME and sodium dodecyl sulfate prior to polyacrylamide gel electrophoresis on a gel containing urea and SDS. The PNPase appeared as two distinct bands, the less intense and slower-moving having a M.W. greater than the native gamma globulin marker (1.5×10^5 daltons), the more intense, faster-moving band being congruent with the bovine serum albumin monomer (7×10^4 daltons). Although no systematic study of the molecular weight of the enzyme was made, the behaviour on G-200 columns was consistent with a molecular weight in the region of 2×10^5 daltons, in agreement with previously determined values (238). Therefore, the slower-moving band is probably the undissociated native enzyme. The SDS results would then suggest that A. vinelandii PNPase is a trimer composed of 7×10^4 dalton subunits. A better estimate of these molecular weights was not possible because of the unavailability at that time of single polypeptide standard

proteins in the region 8-30x10^4 daltons M.W.

d) Catalytic properties

PNPase is considered to have three activities: the phosphorolysis of polyribonucleotides to nucleoside diphosphates; its reverse, the polymerization "incorporation" of nucleoside diphosphates to polyribonucleotides; and what is usually considered to be the equilibrium of the other two, that is the exchange of the terminal phosphate of ADP with inorganic phosphate in the medium. In this section I shall present my data on these three reactions, or more correctly, assay procedures. The fourth reaction, which I mentioned in the introduction, transnucleotidation, I shall treat in a separate section.

i) Incorporation activity

The reaction that I studied in most detail, and on which most of my conclusions are based, is the incorporation activity. The results given in the following sections, therefore, refer mainly to the ADP-incorporation assay, unless otherwise specified. It seemed to me necessary to redetermine the requirement of the pure A. vinelandii PNPase for certain of the variables in the incorporation assay, since some confusion exists in the literature.

The most important kinetic variables in the study of PNPase are the concentrations of ADP and Mg^{++}. The

activity does not depend simply on the absolute concentrations of the two, but rather on their ratio in the assay mixture. (Fig. 3.) We can see that the enzyme has an optimum [ADP] : [Mg^{++}] ratio of around 4.0, irrespective of whether the [ADP] or [Mg^{++}] are varied and the other reagent kept constant. Note that this was a fresh enzyme with no ApA requirement. As I mentioned in the "Introduction," the incorporation activity of A. vinelandii PNPase has been found to depend on the concentration of free nucleoside diphosphate and free magnesium ion, so to be able to construct an intelligible Michaelis-Menten plot one must first determine these values. I did, in fact, attempt to do this, but the procedures found in the literature were not suitable for the concentration range required. I will discuss this fully later.

The incorporation assays were done at pH 9.0 in tris-HCl buffer. This is not the pH maximum, which is actually around pH 9.8 in tris (Fig. 4). Since tris has practically no buffering capacity at this pH, I redetermined the pH curve in tris-HCl and glycine-NaOH buffers and found the optimum in the latter to be pH 9.5. (Fig. 5.) I also observed that whereas the enzyme was slightly stimulated by ApA in tris (<10%) it was slightly inhibited by ApA in glycine-NaOH (<10%). Nevertheless, I continued to carry out my incorporation assays in tris pH 9.0, because tris has some buffering capacity at this pH, and because tris is used

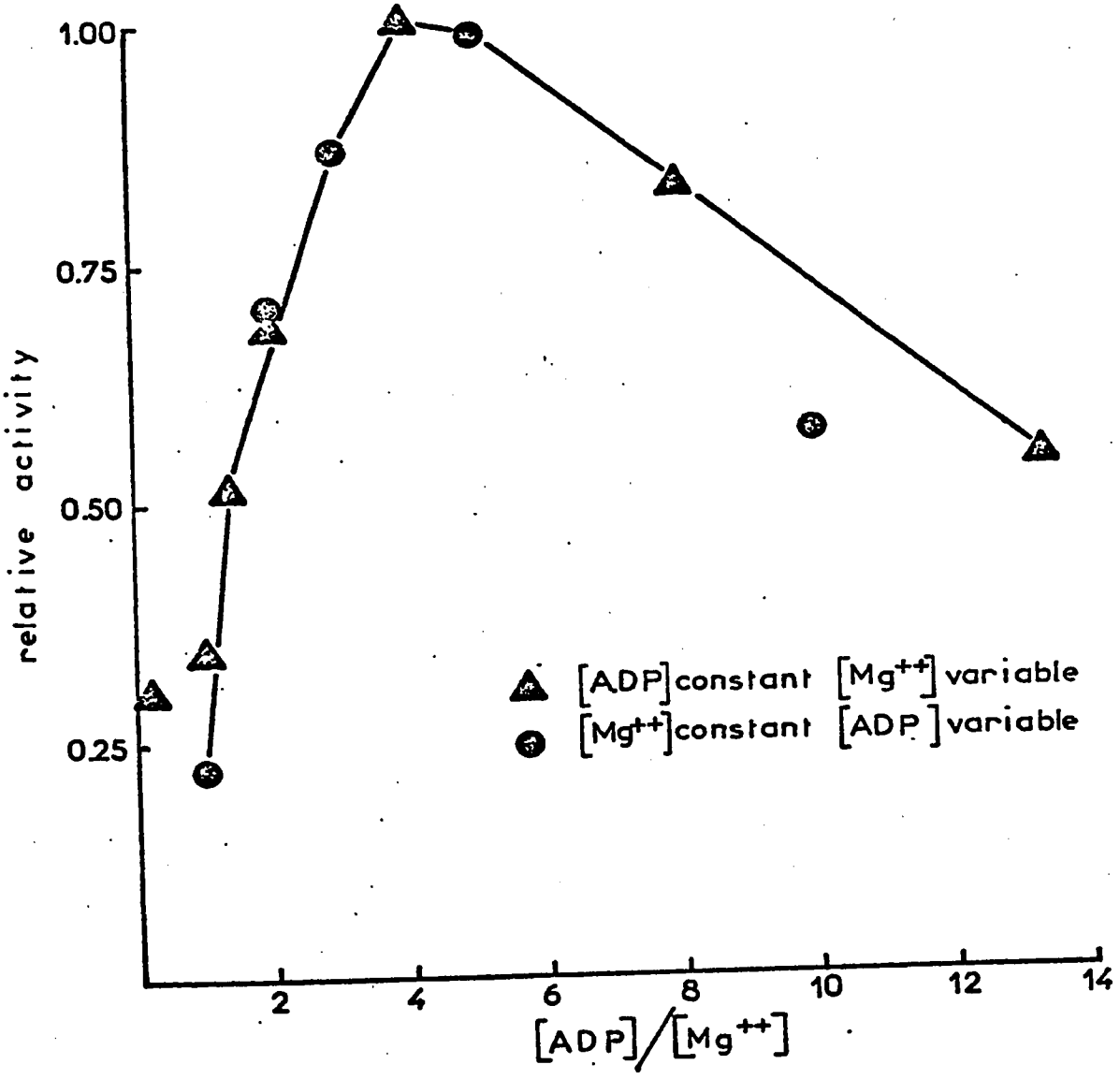


FIG. 3. ADP and Mg^{++} requirement for reduced PNPase

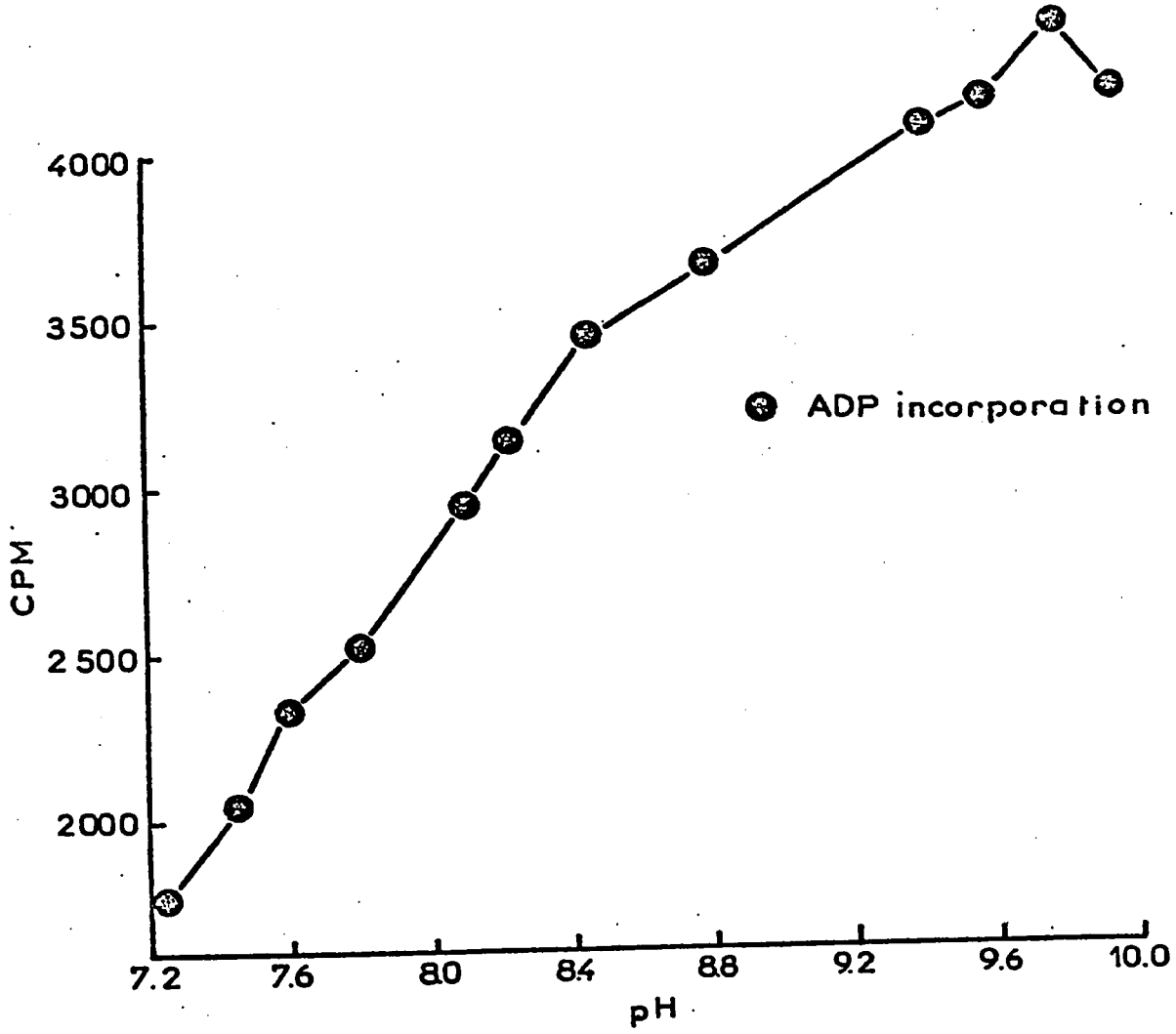


FIG. 4. ADP-incorporation activity as a function of pH

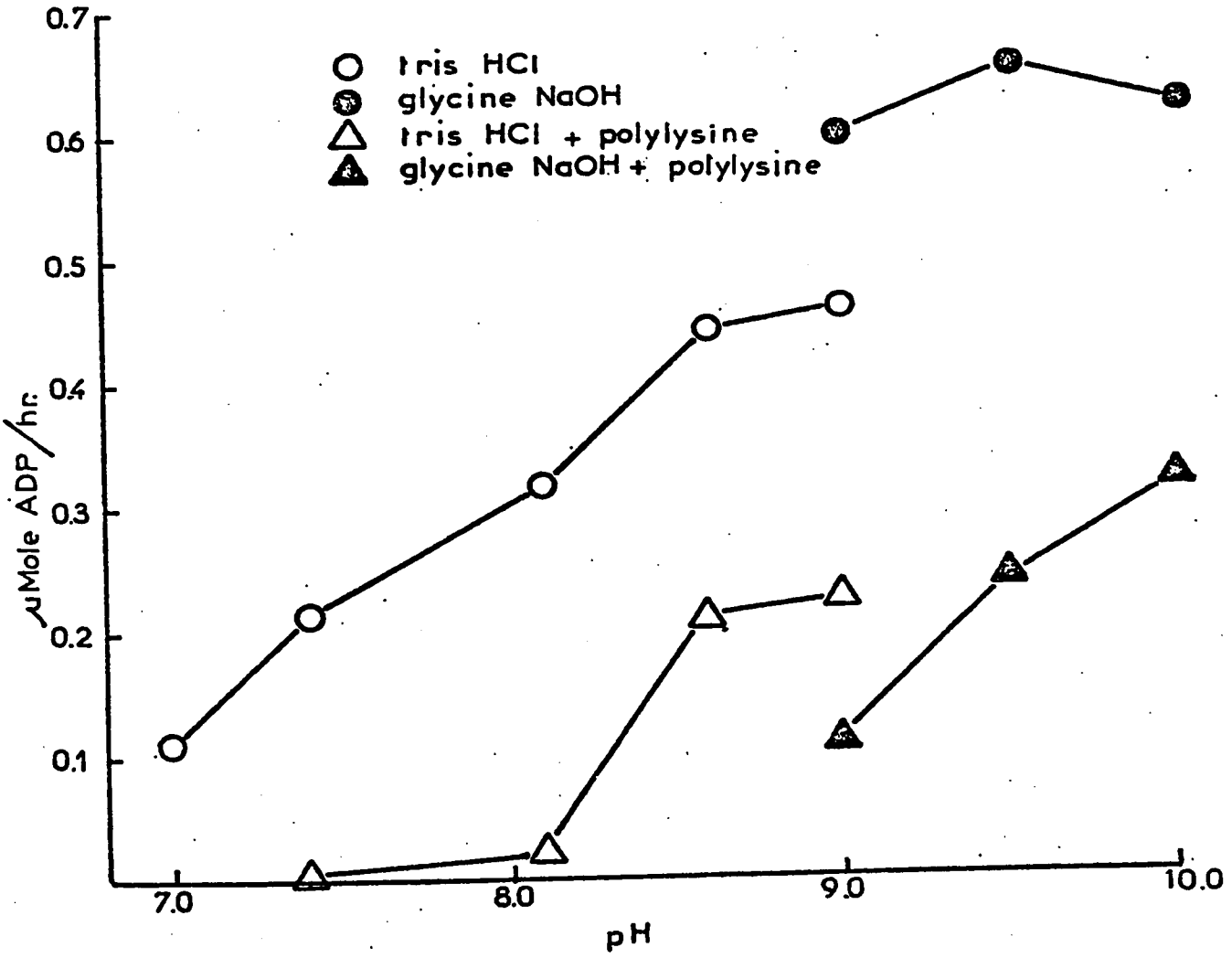


FIG. 5. pH curve of ADP-incorporation activity in optimal conditions, and effect of polylysine

in virtually all published purification and assay procedures for bacterial PNPase.

The ADP-incorporation activity is linear with time up to an incorporation of some 15% of the ADP in the reaction mixture (Fig. 6). The pure enzyme preparation used in this experiment was freshly prepared and was stimulated less than 6% by ApA in the normal assay. The experiment shown in Figure 6b contained four times as much enzyme as that shown in Figure 6a. The procedure was to pre-incubate the enzyme and other ingredients of the assay mixture separately at 37°, and to add the two together at zero time. Appropriate samples were removed at the indicated times, added to cold 7% perchloric acid and the samples worked up as usual. The specific activity of the ADP in the reaction mixture was 15,500 cpm/μmol with a total of 4 μmol of ADP. As we can see in Figure 6a the enzymatic activity is linear with time and in Figure 6b with more enzyme, that the linearity extends to almost half a micromole of ADP incorporated into polyA, i.e. about 15% completion. As a first approximation of the best straight line fitting the points I have drawn in the simple average of the slopes from the origin to each of the points (in Fig. 6b disregarding the values after 10 min).

It seems clear that there is no lag phase either in the presence or absence of ApA, and that in fact, there is no ApA requirement. Although freshly prepared PNPase sometimes shows a primer requirement, this is not consistent and

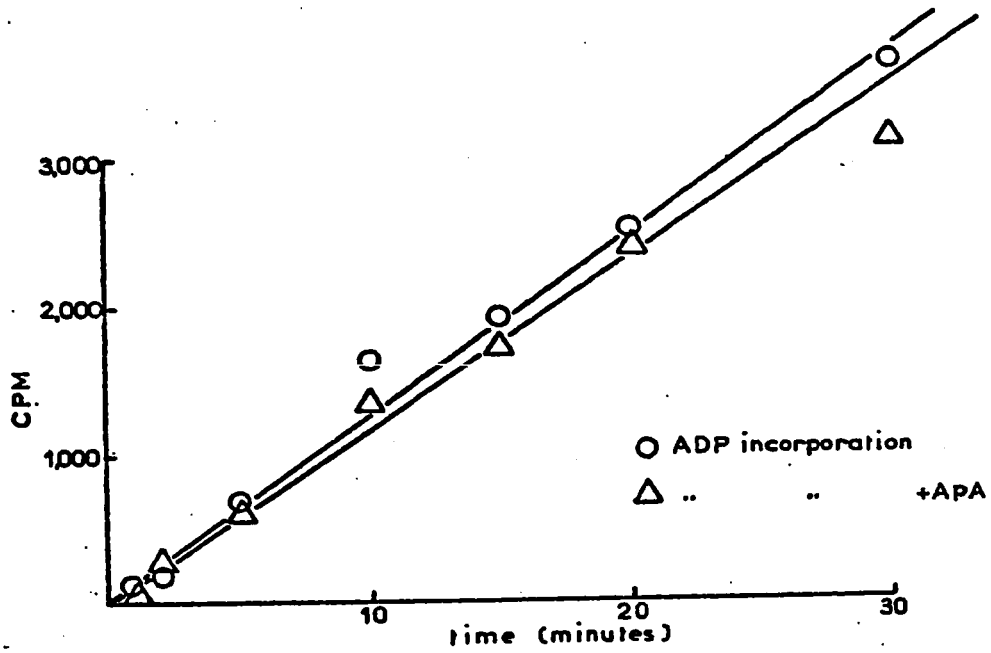


FIG. 6 (a)

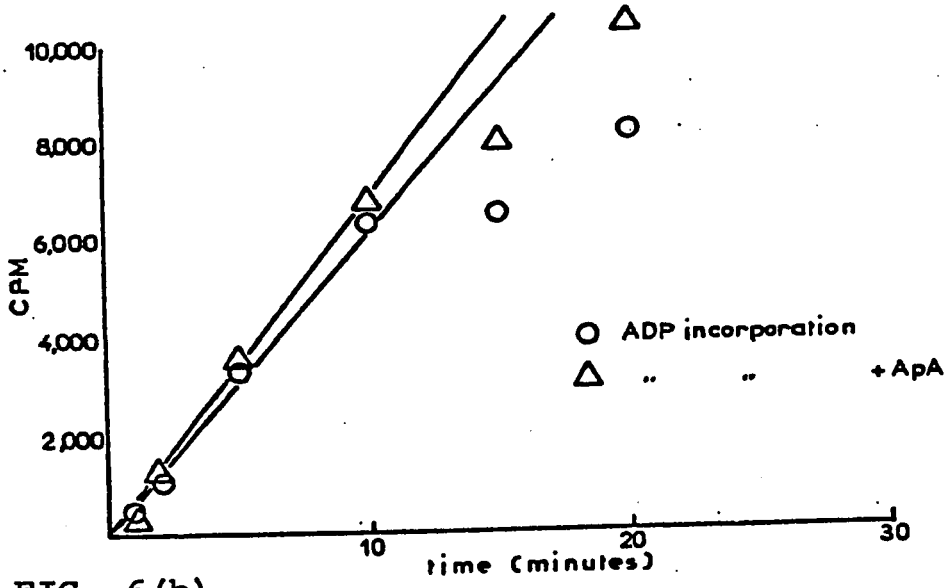


FIG. 6 (b)

FIG. 6. Time curves of ADP-incorporation activity

sometimes there is even inhibition by ApA as in Figure 6a. The inhibition or stimulation in these cases is less than 10%, which I judge to be within the experimental error. The activity is proportional to enzyme concentration, the slope in Figure 6b being 560 cpm/min, exactly four times the slope of 140 cpm/min in Figure 6a.

Bovine serum albumin (BSA) stimulates the activity of the pure enzyme. With enzyme preparations of specific activity greater than 2,000 units/mg (i.e. approximately 25% pure?) this stimulation was at least 15% and could be as high as 25%. The effect was independent of the effect of ApA and indeed the effects were additive. 5 µg of BSA in the assay mixture was sufficient to give maximal stimulation and additional amounts up to 100 µg did not give additional stimulation, nor did they inhibit the activity. The stimulation was more noticeable with small amounts of enzyme, so it is obvious that BSA improves the proportionality of the assay with respect to enzyme concentrations. For this reason 5 µg of BSA were routinely included in all assays, unless specifically stated otherwise.

A quick glance at the constituents of the incorporation assay mixture shows that the total concentration of the salts (tris; $MgCl_2$) is 160 mM. This is not the optimum, since additional NaCl stimulates 10%, but higher concentrations inhibit the assay, and the inhibition is total in 1.5 M NaCl (Fig. 7).

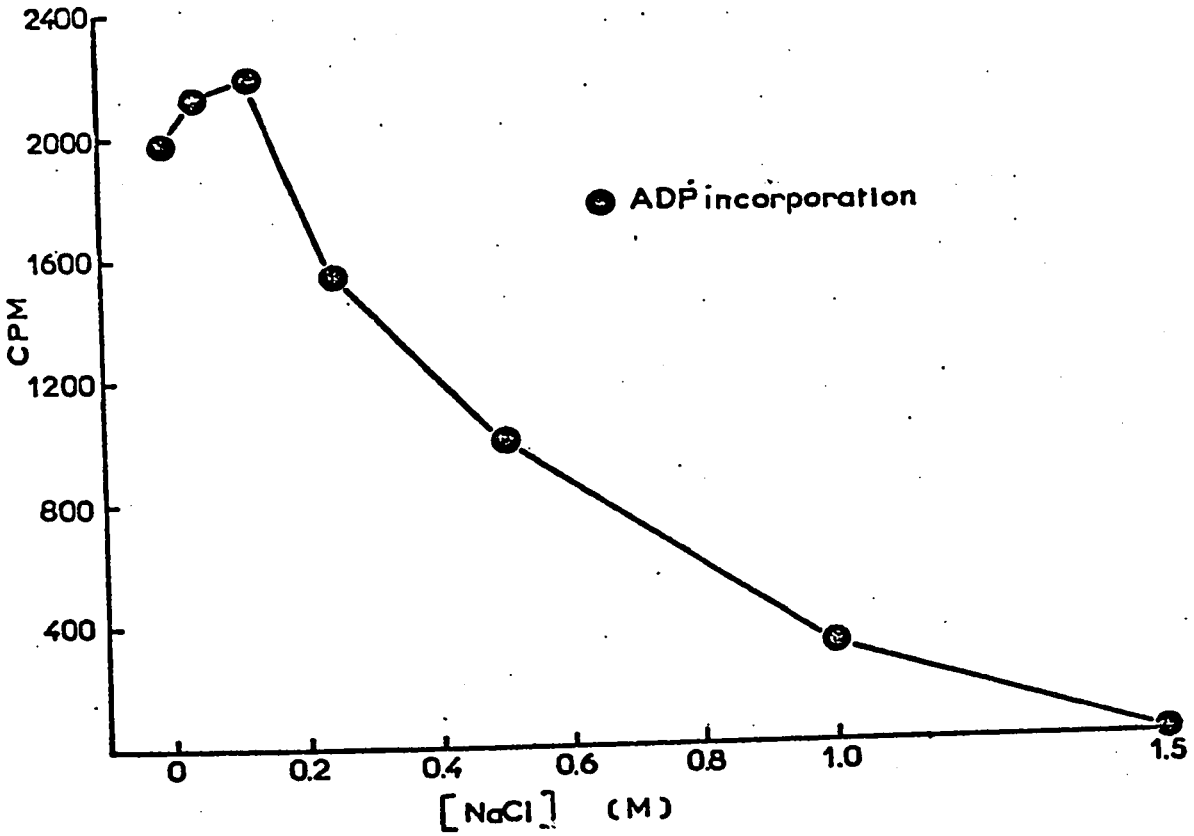


FIG. 7. Effect of NaCl on ADP-incorporation activity

In connection with the salt stimulation, it has been observed that with Cl. perfringens PNPase, polylysine dramatically stimulates the ADP-incorporation activity, and that this effect is partially spared by NaCl. This is not the case with A. vinelandii PNPase (Fig. 5), since polylysine severely inhibits the activity at all pH values. Further, even in the optimal conditions of assay for Cl. perfringens PNPase, polylysine does not stimulate the A. vinelandii enzyme (Fig. 8). In short, A. vinelandii PNPase resembles the M. luteus PNPase, rather than the Cl. perfringens PNPase.

With respect to the mechanism of action, cyclic AMP is not incorporated into an acid-insoluble precipitate. It does, however, stimulate the ADP incorporation assay some 20% at 7 mM, but inhibits the CDP assay 10% at this concentration. On the other hand, both 3'UMP and 3'CMP inhibit the ADP incorporation assay 15% at 25 mM and 40% at 125 mM. The effect is not reversed by ApA.

ii) Exchange and phosphorolysis activities

I did not carry out many studies on the exchange reaction, preferring to concentrate on the other two assay procedures. I did, however, confirm that the enzyme does catalyze this reaction. Of the assay procedures given in the literature, I chose the one given by Thang (214), since it was the most recent one reported for A. vinelandii PNPase and because it gave 1.8 times the activity of the method

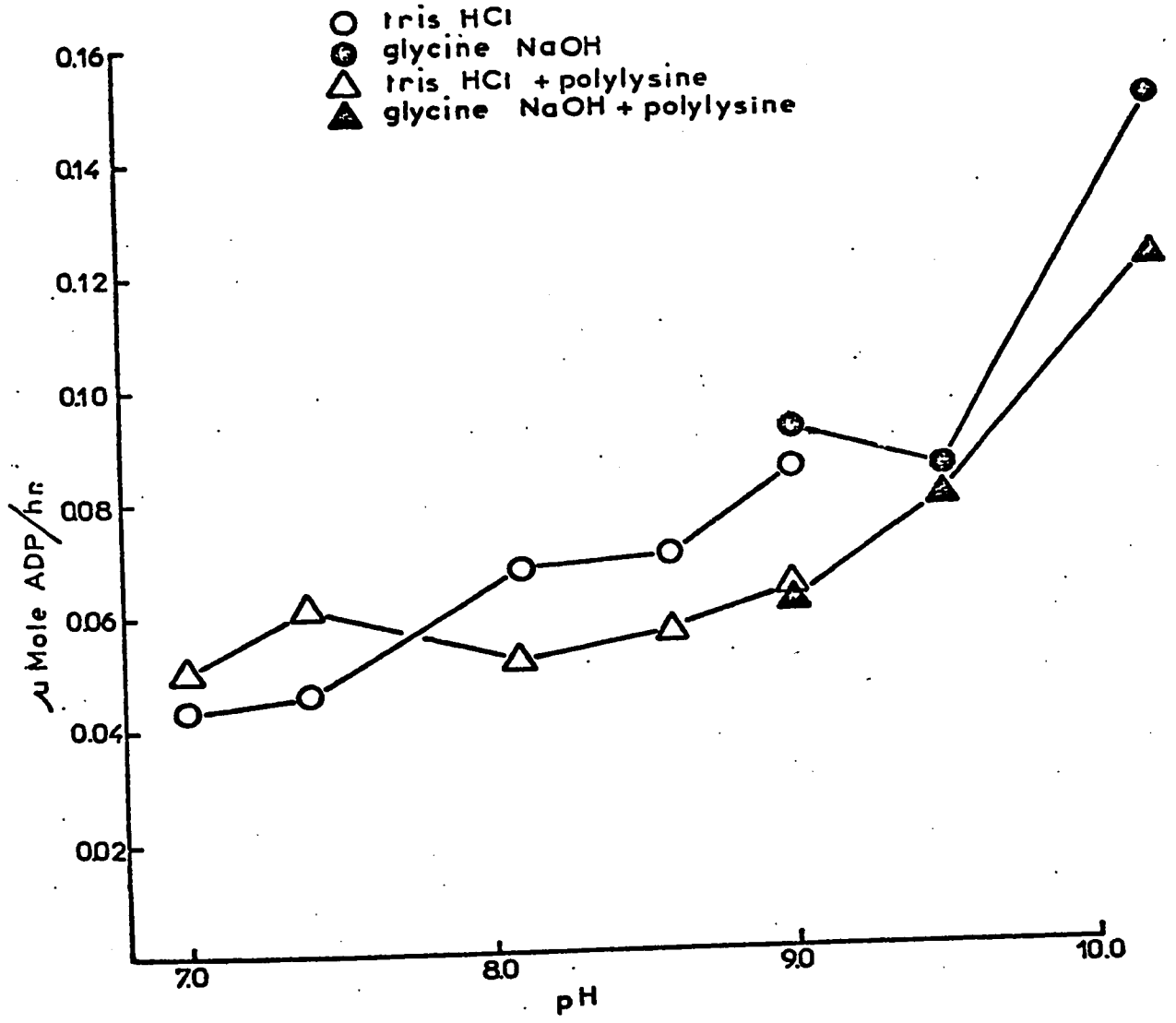


FIG. 8. pH curve of ADP-incorporation activity in non-optimal conditions, and effect of polylysine

described by Grunberg-Manago (88) when the activities were calculated according to the expression given in the "Introduction." I monitored one preparation by exchange activity and found that it roughly parallelled the ADP and phosphorolytic activities (Table 16). I should mention that this is the same preparation shown in Table 14, but in more detail. It is clear that, at least as far as these assay procedures are concerned, the ADP and CDP activities are roughly twice the exchange activity, which is in turn twice the phosphorolysis activity. I do not consider these results to be of any particular significance, since they obviously depend on the assay conditions chosen. The important point to note is that the exchange activity is not stimulated by ApA, except perhaps in the early stages of the purification.

The phosphorolysis assay on occasion, gives puzzling results (Table 16). In this preparation there are seemingly arbitrary gains and losses of activity at various stages and the overall yield of the preparation is much worse than when measured by nucleoside diphosphate incorporation. The phosphorolytic activity varies with the polyA preparation used, both from preparation to preparation, and less markedly from day to day. It is very difficult to accurately pipette small volumes of polyA solution (20 mg/ml) because of its high viscosity and this could be a factor. I have also found that polyA solutions, which have been repeatedly frozen

TABLE 16

Relative purifications of various activities of PNPase

Enzyme Fraction		ADP-incorp (units)		CDP-incorp (units)		Exchange (units)		Phosphorolysis (units)
		-ApA	+ApA	-ApA	+ApA	-ApA	+ApA	
Crude	Total units	43,600	62,400	83,200	118,300	75,400	88,000	43,500
	Spec Act	2.1	3.0	4.0	5.6	3.6	4.2	2.1
AS	Total units	62,300	64,800	45,100	58,200	97,500	88,600	59,000
	Spec Act	5.7	5.9	4.1	5.3	8.0	8.1	5.4
DEAE I	Total units	62,300	69,200	84,300	136,000	38,500	41,800	18,100
	Spec Act	240	260	320	570	150	160	65
G-200	Total units	50,400	57,200	54,500	64,000	30,300	26,100	20,400
	Spec Act	1,250	1,300	1,360	1,600	750	650	510
DEAE II	Total units	60,000	60,000	30,800	47,200	25,200	25,200	15,100
	Spec Act	7,300	7,300	3,900	6,000	3,200	3,200	1,900
QAE	Total units	41,200	35,300	26,000	37,400	21,700	19,300	10,100
	Spec Act	8,800	7,500	5,500	8,000	4,600	4,100	2,200

and thawed, or even stored in basic solutions for long periods (70), are much poorer substrates for phosphorolysis, than freshly prepared solutions. Although I have monitored many of my experiments by the phosphorolysis assay, in all cases the assays are internally consistent, since they were performed on a single day with a single polyA preparation. One last point to note in passing is, that I have found that 3'-5' cyclic AMP is not phosphorolyzed by A. vinelandii PNPase, nor does it inhibit the phosphorolysis of polyA.

iii) Transnucleotidation activity

Of extreme interest with respect to the mechanism of reaction of PNPase is the report of Singer et al. (199), concerning the transnucleotidation activity of A. vinelandii PNPase, which I have discussed in the Introduction. I carried out a few preliminary experiments to determine whether the enzyme did in fact catalyze this reaction, and if so, under what conditions. Of the two pH values tried, pH 9.0 was far better than pH 8.2 and showed quite conclusively that transnucleotidation had taken place. I investigated the reaction further using an adaptation of the method described by Thach (210), for the development of the chromatograms. Chromatography was carried out at room temperature on Whatman 3 MM paper using 7 parts 1M ammonium acetate pH 7.2 to 3 parts 95% ethanol, as an eluting solvent. The chromatograms were pre-equilibrated one hour with the

solvent vapour before the start of development. Solutions were applied 10 cm from one end of the paper, leaving approximately 45 cm for separation of the nucleotides. Development of the chromatograms was for 18-22 h, at which latter time a spot identified as 5'AMP was approaching the end of the paper. Under these conditions ADP and ATP travelled faster than 5'AMP (which was not separated from 3'AMP). The oligonucleotide series ApA through $(Ap)_5A$ travelled progressively slower and were well separated from each other and from the origin.

For the first experiment nine test tubes were set up, containing the reagents listed in Table 17. In addition to the reagents listed, each tube contained: tris-HCl buffer pH 9.0, 0.6 µmol; $MgCl_2$, 0.02 µmol; EDTA, 0.003 µmol. Total volume of each solution was 60 µl. The tubes were incubated at 37° for one hour, then placed in ice during the time the solutions were spotted. 40 µl of each solution was spotted in the appropriate position at the origin and the chromatogram was developed for 22 h, as described above. The chromatogram was then air-dried and the spots located under U.V. light. Results are shown in Figure 9.

As we can see from the AMP marker spots, the chromatogram is slightly skewed, but the separation of the spots is sufficient to identify them. Tubes 1-4 did not contain enzyme and therefore served as markers. Tube 5 (5'AMP), tube 6 (ApA), and tube 7 ($(Ap)_2A$), do not show any

TABLE 17

Experimental protocol for transnucleotidation I
(see text for further details, results in Fig. 9)

Nucleotide	Tube								
	1	2	3	4	5	6	7	8	9
ADP	1 µmol	-	-	-	-	-	-	-	-
5'AMP	0.1 µmol	-	-	0.1 µmol	0.1 µmol	-	-	-	-
ApA	-	40 µg	-	-	-	40 µg	-	-	-
$(Ap)_2A$	-	40 µg	-	-	-	-	40 µg	-	-
$(Ap)_3A$	-	-	40 µg	-	-	-	-	40 µg	-
$(Ap)_4A$	-	-	40 µg	-	-	-	-	-	40 µg
$(Ap)_5A$	-	-	-	40 µg	-	-	-	-	-
PNPase	-	-	-	-	13 ADP units	13 ADP units	13 ADP units	13 ADP units	13 ADP units

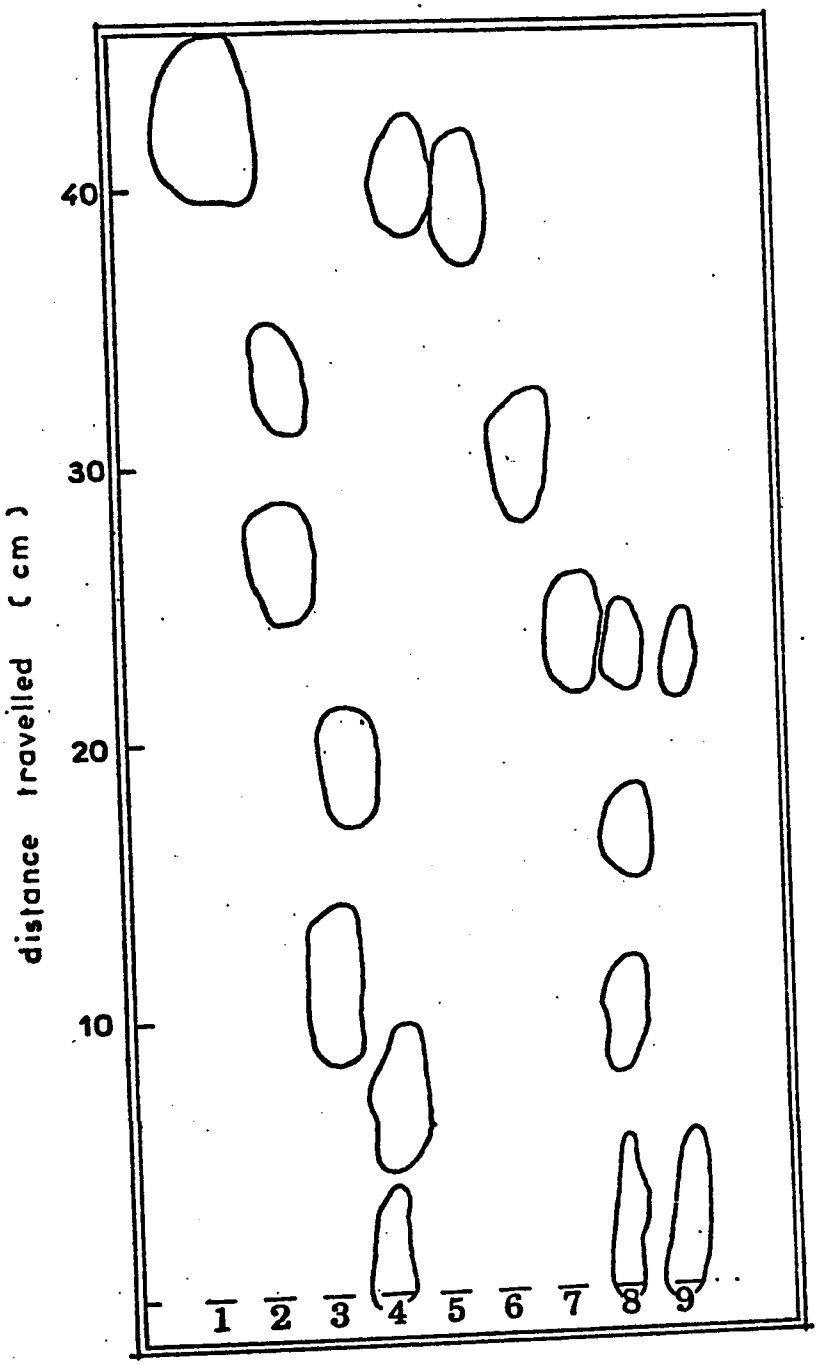


FIG. 9. Results of transnucleotidation experiment I (viz. Table 17)

signs of transnucleotidation, but tube 8 ($(Ap)_3A$) definitely shows spots other than substrates, identified as $(Ap)_2A$, $(Ap)_4A$ and even $(Ap)_5A$ and other higher homologues. Tube 9 shows $(Ap)_2A$ and $(Ap)_5A$ and higher homologues. Moreover, tube 4, although it did not contain enzyme, seems to show a higher homologue of $(Ap)_5A$. This was shown to be an artefact in a separate experiment. It is obvious that in tubes 8 and 9 the transnucleotidation reaction has occurred.

The experiment was repeated in slightly different conditions to confirm the occurrence of transnucleotidation. Again, nine test tubes were set up, containing the reagents listed in Table 18. In addition, tubes 2, 3, 4, 5, 6, 7 and 8 contained: tris-HCl buffer, pH 9.0, 0.6 µmol; $MgCl_2$, 0.02 µmol; EDTA, 0.003 µmol. These tubes contained a final volume of 70 µl of which 60 µl were spotted after incubation. Tubes 1, 5 and 9 contained: tris-HCl buffer pH 9.0, 3.0 µmol; $MgCl_2$, 0.15 µmol; EDTA, 0.022 µmol. Note also that these tubes did not contain PNPase. Rather, the enzyme was spotted after incubation and spotting of the other reagents. Similarly tubes 3, 4, 5, 6, 7 and 8 did not contain 5'AMP, which was spotted after incubation and spotting of other reagents, except in the case of tube 6. Other experimental details were exactly as in the previous experiment, except that development was for 18 h.

The results (Fig. 10) are clear-cut. As expected, 5'AMP alone, and ApA alone do not serve as substrates for

TABLE 18

Experimental protocol for transnucleotidation II
(see text for further details, results in Fig. 10)

Nucleotide	Tube 1	2	3	4	5	6	7	8	9
5'AMP	1.0 µmol	1.0 µmol	0.5 µmol*	0.5 µmol*	0.5 µmol*	-	0.5 µmol*	0.5 µmol*	1.0 µmol
ApA	100 µg	-	100 µg	-	100 µg	-	-	-	100 µg
$(Ap)_2A$	100 µg	-	-	100 µg	100 µg	-	-	-	100 µg
$(Ap)_3A$	100 µg	-	-	-	100 µg	100 µg	-	-	100 µg
$(Ap)_4A$	100 µg	-	-	-	100 µg	-	100 µg	-	100 µg
$(Ap)_5A$	100 µg	-	-	-	100 µg	-	-	100 µg	100 µg
PNPase	26 ADP units*	26 ADP units	26 ADP units	26 ADP units	26 ADP units*	26 ADP units	26 ADP units	26 ADP units	26 ADP units*

*spotted after incubation and spotting of other reagents

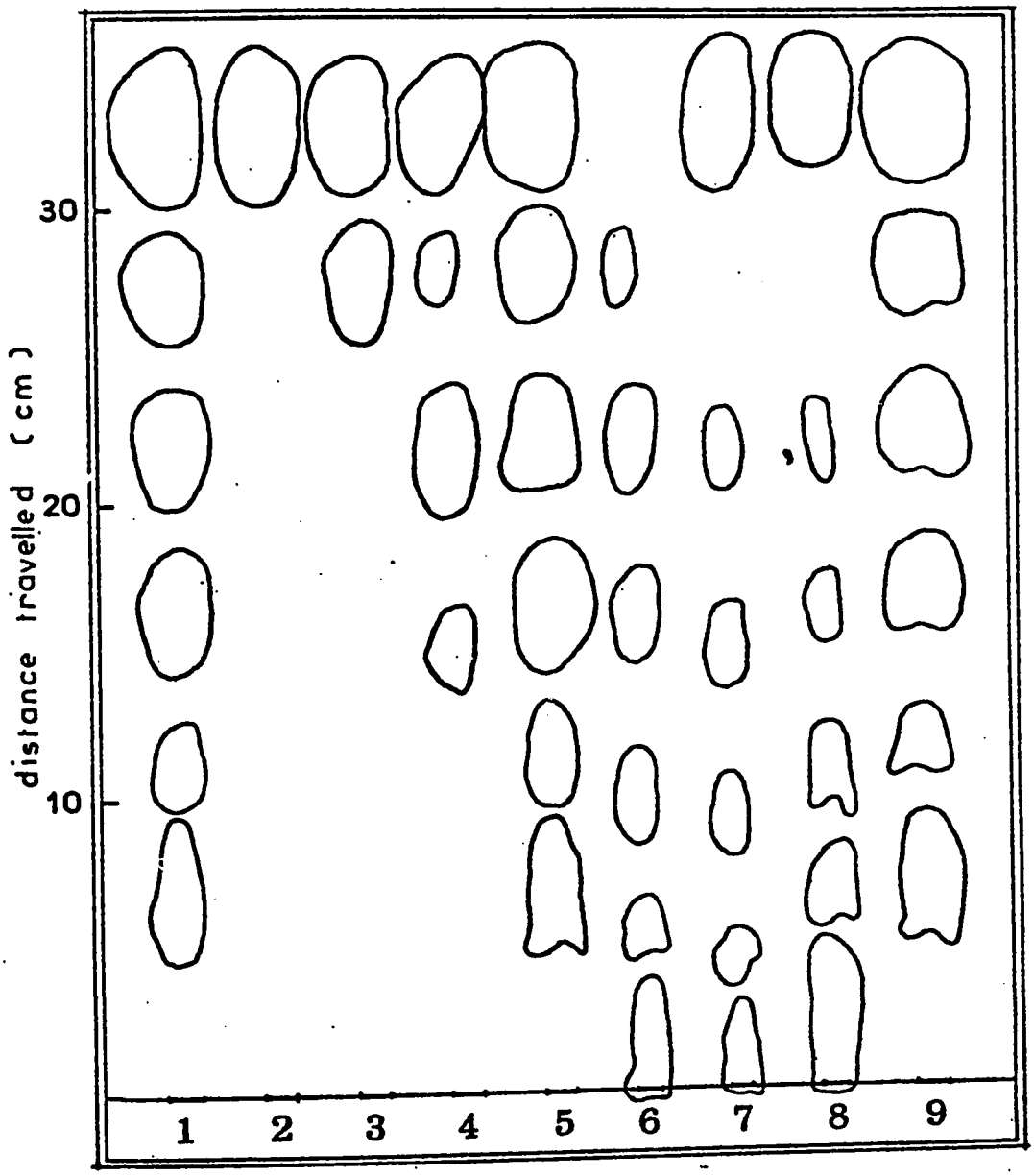


FIG. 10. Results of transnucleotidation experiment II (viz. Table 18)

the transnucleotidation reaction. $(Ap)_2A$ is not a good substrate, but weak spots appeared for the next higher and lower homologues. $(Ap)_3A$ through $(Ap)_5A$ are excellent substrates, showing spots for both the higher and lower homologues, the spots being progressively weaker farther from the primary substrate. I consider this result to be conclusive proof that A. vinelandii PNPase catalyzes the transnucleotidation reaction.

The question remains, however, if 5'AMP might not be involved, adding directly to the oligonucleotide substrate. To test this hypothesis the following experiment was carried out. Seven tubes were set up, containing the reagents listed in Table 19. In addition the tubes contained: tris-HCl buffer, pH 9.0, 0.6 µmol; $MgCl_2$, 0.02 µmol; and EDTA, 0.003 µmol. Incubation was at 37° for one h as before. Each tube contained 0.1 ml, of which 90 µl were spotted. Development was for 18 h, as previously. After development, the spots were located and identified under U.V. light, the chromatogram was cut up in an appropriate pattern, each piece was finally shedded, placed in a standard scintillation mix, and counted in a scintillation counter.

Results are summarized in Table 20. The numbers refer to the counts per minute for each sample recorded by the scintillation counter. The crosses refer to the relative size and intensity of the spots observed under U.V. light. As expected, some of the compounds served as

TABLE 19

Experimental protocol for transnucleotidations III
(see text for further details, results in Table 20)

Nucleotide	Tube						
	1	2	3	4	5	6	7
5'AMP	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol
[^{14}C]-5'AMP	-	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm
ApA	50 µg	-	100 µg	-	-	-	-
$(Ap)_2A$	50 µg	-	-	100 µg	-	-	-
$(Ap)_3A$	50 µg	-	-	-	100 µg	-	-
$(Ap)_4A$	50 µg	-	-	-	-	100 µg	-
$(Ap)_5A$	50 µg	-	-	-	-	-	100 µg
PNPase	-	26 ADP units	26 ADP units	26 ADP units	26 ADP units	26 ADP units	26 ADP units

TABLE 20

Results of transnucleotidation III

(see Table 19)

Nucleotide	Tube						
	1	2	3	4	5	6	7
AMP	236 XXX	56,278 XXX	48,652 XXX	48,090 XXX	50,669 XXX	51,010 XXX	50,443 XXX
ApA	64 XXX	770 -	1,684 -	997 X	1,001 X	771 -	489 -
$(Ap)_2A$	52 XXX	168 -	166 -	395 XXX	141 XXX	129 XX	121 -
$(Ap)_3A$	50 XXX	108 -	110 -	134 X	85 XX	90 XX	88 X
$(Ap)_4A$	- XXX	- -	- -	- -	- XX	- XX	- XX
$(Ap)_5A$	- XXX	- -	- -	- -	- X	- X	- XX

substrates for transnucleotidation. From the data shown one might suspect that [^{14}C]-5'AMP had been incorporated into ApA in tubes 3, 4 and 5, and into $(Ap)_2A$ in tube 4. However, there is much trailing of radioactivity from the 5'AMP spots, and even cross-contamination to the sides, so it is difficult to draw firm conclusions. The experiment was repeated according to the protocol in Table 21. In all other respects the experimental procedure was identical to that in the preceding experiment. Results are summarized in Table 22. As expected, transnucleotidation took place only in tube 7 and it is obvious that no [^{14}C]-5'AMP was incorporated into the oligonucleotides. In view of the result for tube 1, which contained no enzyme, the high radioactivity in the ApA and $(Ap)_2A$ spots for tube 5, which contained ApA and enzyme, can be dismissed as non-specific absorption of radioactivity to the paper. The only alternative possibility is that [^{14}C]-5'AMP can be incorporated into ApA non-enzymatically, but in this case tube 4 should also show the effect. I think it safe to say on the basis of these results that free 5'AMP does not in any way serve as a substrate for the transnucleotidation reaction.

Before concluding this section, I should mention several additional observations not reported above. The chromatographic patterns were identical whether or not the nucleotides were incubated at 37° prior to spotting, in the absence of enzyme. Similarly, the presence or absence of

TABLE 21

Experimental protocol for transnucleotidation IV
(see text for further details, results in Table 22)

Nucleotide	Tube						
	1	2	3	4	5	6	7
5'AMP	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol	0.5 µmol
[^{14}C]-5'AMP	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm	$5x10^4$ cpm
ApA	50 µg	-	-	100 µg	100 µg	-	-
$(Ap)_2A$	50 µg	-	-	-	-	100 µg	100 µg
$(Ap)_3A$	50 µg	-	-	-	-	-	-
PNPase	-	-	27 ADP units	-	26 ADP units	-	26 ADP units

TABLE 22

Results of transnucleotidation IV

(see Table 21)

Nucleotide	Tube						
	1	2	3	4	5	6	7
5'AMP	48,731 XXX	48,500 XXX	49,621 XXX	45,840 XXX	45,105 XXX	52,005 XXX	46,826 XXX
ApA	1,783 XXX	450 -	555 -	529 XXX	1,629 XXX	760 -	822 X
$(Ap)_2A$	232 XXX	130 -	123 -	128 -	217 -	131 XXX	163 XXX
$(Ap)_3A$	103 XXX	73 -	85 -	80 -	88 -	89 -	89 XX

the inorganic constituents of the reaction mixture (tris, Mg^{++}, EDTA) did not change the mobility of the nucleotides. No reaction was observed in the absence of either Mg^{++} or enzyme. In all cases where enzyme was present at the origin before development, two additional spots were observed: a brown spot, invisible under U.V. light, which remained at the origin during development; and spot fluorescing yellow under U.V. light, which travelled with the solvent front. These are obviously constituents of the enzyme preparation.

e) Primer requirement and effect of aging

A. vinelandii PNPase, in common with the M. luteus and E. coli enzymes, has a primer requirement in the incorporation assay. It is more like the former than the latter in that this requirement does not appear immediately in the purified enzyme and develops only after an appreciable length of time. (Fig. 11.) The first 45 days on the time axis represent the length of time taken to purify the enzyme and the points are the various purification steps. At day 45 the enzyme has a specific activity of 6,000 units/mg, and no primer requirement. At various times thereafter the enzyme was assayed with and without ApA, giving the results shown. The increase is not regular with time, and in fact, on any given day can vary as much as 50%. However the trend is clear; the enzyme is increasingly stimulated by ApA with

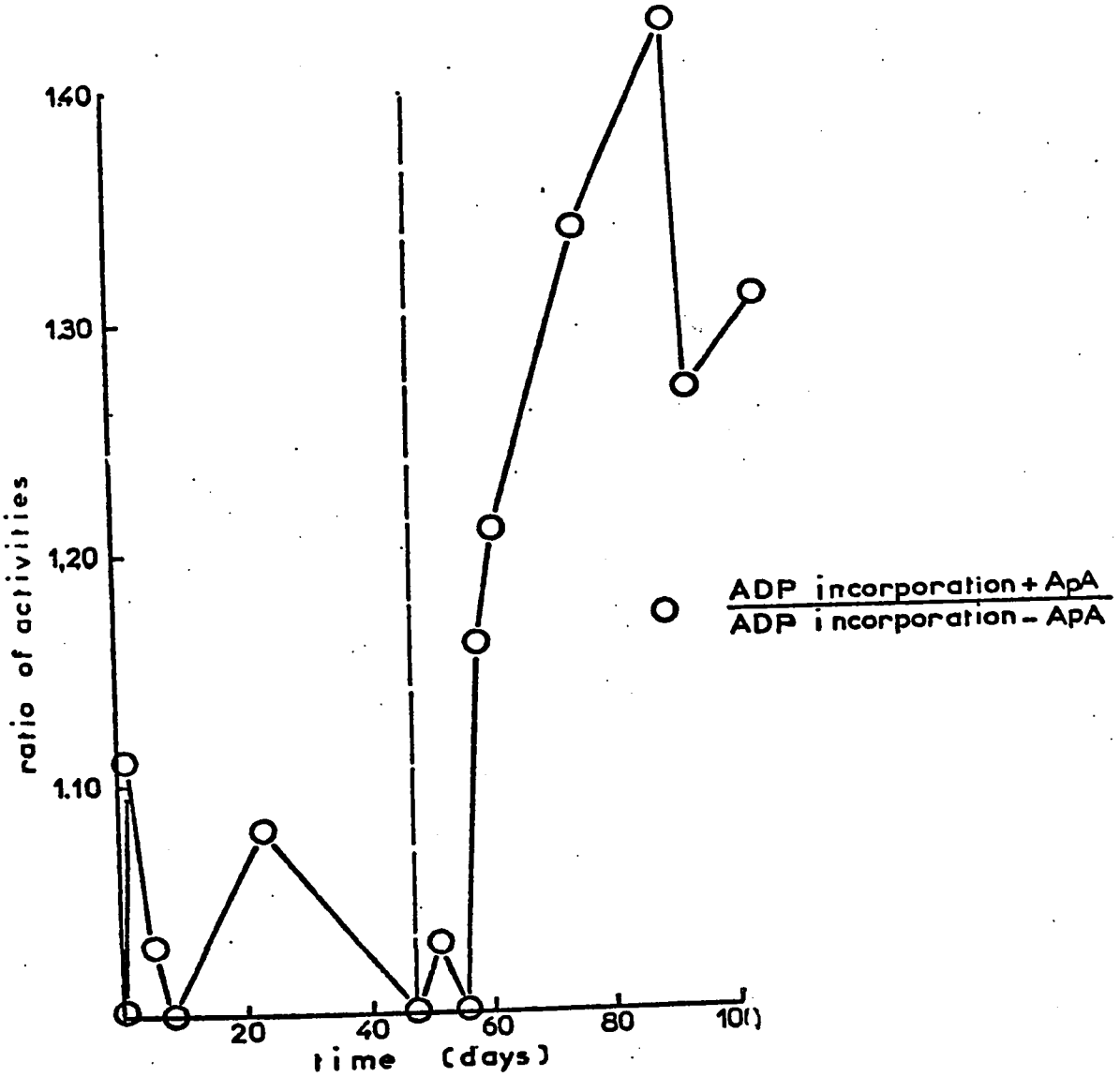


FIG. 11. Increase in ApA-stimulation of ADP-incorporation activity with age

longer storage times in TEB 8.2 buffer. Some irreversible losses also occur. Taking day 57 as a base of 100%, on day 80 there remained 93% of the activity, on day 86, 88%, and on day, 100, 75%, all measured in the presence of ApA.

The effect is not quantitatively reproducible with other preparations of the enzyme, although qualitatively similar results are always observed. For instance, another preparation was 60% stimulated by ApA after 4 months, another 8% after 2 months and another 20% after 3 months. Further, there is no obvious way to accelerate this natural increase in primer requirement in a reproducible manner, although it did seem that one preparation, which contained a relatively high amount of nucleic acid (8%) did not develop the primer requirement as quickly as the usual preparations (7% nucleic acid). Treatment with trypsin does increase the primer requirement, as I shall discuss elsewhere, but this causes other changes, which are quite unlike the aging phenomenon.

Another important consideration is that the stimulation by ApA of the CDP-incorporation activity is always higher than that for ADP. Even in a freshly prepared enzyme preparation with no ADP-primer requirement the CDP assay is usually stimulated some 40% by ApA. In the case of the preparation shown in Figure 11, the CDP activity of the fresh enzyme on day 45 was stimulated 53%, on day 79, 114%, and on day 104, 162%. On another occasion, I observed

stimulation of 140% in an aged preparation, whose ADP activity was stimulated only 10% by ApA.

The changes in primer requirement are accompanied by changes in the electrophoretic properties of the enzyme. In the particular case of the preparation shown in Figure 11, the results are shown in Figure 12. On day 45 the fresh enzyme had only a single heavy band (other than the slow-moving non-protein "anomalous" band). By day 62 a second, very light band had appeared with a slightly higher mobility than the original band. In Figure 12 the separation between these two bands is exaggerated, since the second band actually appears as a shoulder of the first one, and can barely be resolved by eye. In no case was this second band resolved by densitometry. Between days 62 and 90, the first band becomes progressively less intense, while the second band becomes more intense. However, I have never obtained an aged preparation which contained only the second band. These two bands could also be detected on gels stained for ADP-incorporation activity, although in this case, their relative intensities remained approximately the same between days 62 and 90. Neither of the bands had an absolute primer requirement, since they were both evident whether or not ApA was present in the assay medium (with much lower intensity, of course, in the absence of ApA). The Methylene Blue staining procedure suggested that nucleic acid was present in both bands, but they were not resolved by this method,

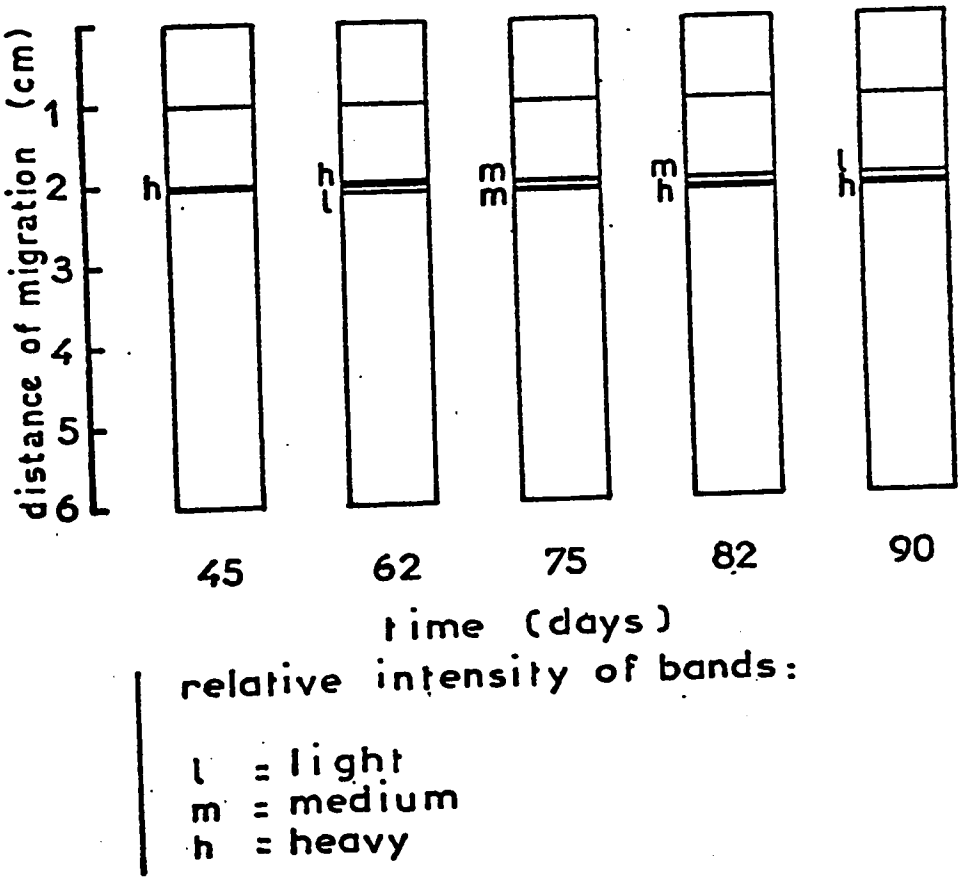


FIG. 12. Increase in electrophoretic mobility with age

which gives more diffuse bands than the other staining procedures.

In all the assays in this work, whenever ApA was included in the assay, the standard amount used was 100 µg ApA in the assay. When 200 µg was used, the stimulation was invariably less than with 100 µg, whether the enzyme had a large or a small primer requirement, or whether the ADP- or CDP-incorporation activities were measured. Further, there seemed to be no specificity with respect to the nature of the primer, since CpC stimulated both ADP and CDP assays as well as did ApA (Table 23). On the other hand, polyA (100 µg) was only half as effective as ApA as a primer.

Since most of the experiments in this work were done with enzyme that was only slightly, if at all, stimulated by ApA, I did not investigate the reported lag phase in incorporation activity. Nevertheless, a very short lag phase did appear on occasion with A. vinelandii PNPase and was not abolished by addition of ApA to the assay (Fig. 18).

f) Trypsin-treatment

The original project which was to have formed the bulk of this thesis, was the investigation of the action of proteolytic enzymes on the activity of polynucleotide phosphorylase from various bacterial sources, continuing the early work of Olmstead and Lowe (168), and Fitt and Fitt (65). It was during the course of experiments on the action

TABLE 23

Effect of NpN primer on incorporation activities

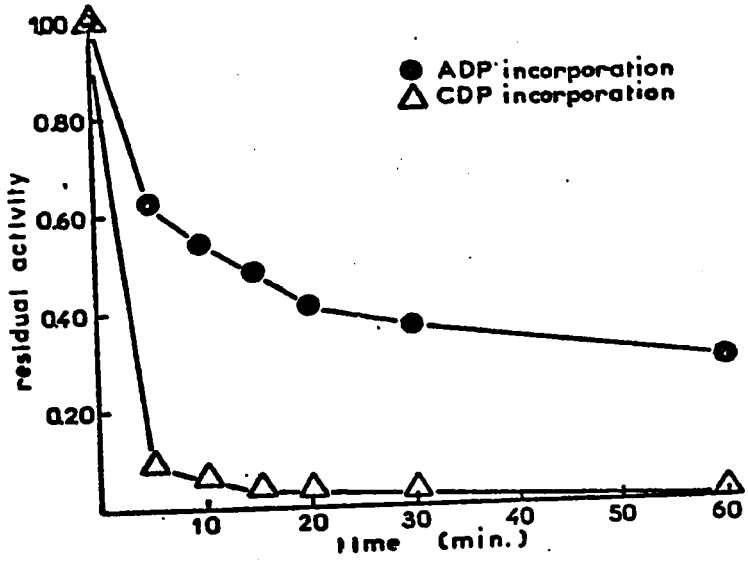
Primer	ADP-incorporation	CDP-incorporation
	(relative to assay without primer)	
ApA (10 µg)	1.01	1.41
ApA (20 µg)	1.02	1.56
CpC (100 µg)	1.05	1.35
CpC (200 µg)	1.00	1.30

of trypsin on A. vinelandii PNPase that the reversible oxidation phenomenon was observed. Klee and Singer (130) had reported and Fitt et al. (67) had confirmed that BME reversed the effect of trypsin on M. luteus PNPase. This seemed also to be the case for the A. vinelandii enzyme, except that activity recovered from the trypsin-then-BME-treated enzyme was greater than that of the untreated enzyme. Further work on this topic was therefore set aside until the purification and oxidation aspects of the work had been cleared up.

Investigations of the action of trypsin on M. luteus PNPase showed that trypsin had a "differential" effect on the ADP- and CDP-incorporation activities, i.e. that any loss in the ADP-incorporation activity was accompanied by a much greater loss in the CDP-incorporation activity. My earliest experimental data with impure enzyme preparations seemed to confirm this both for E. coli PNPase (Fig. 13a) and for A. vinelandii PNPase (Fig. 13b). The effect was qualitatively similar for preparation at various stages of purification from both these sources. A similar effect was seen with chymotrypsin digestion of impure E. coli PNPase, although the "differential" effect was not as dramatic.

Figure 14 shows typical results of a trypsin digestion of a pure A. vinelandii PNPase. The procedure, briefly, was to pre-incubate equal portions of PNPase (0.3 mg/ml, ADP spec. act. 6,000 units/mg) in TEB 8.2 buffer, and

FIG. 13(a). Effect of trypsin-treatment on impure *E. coli* PNPase



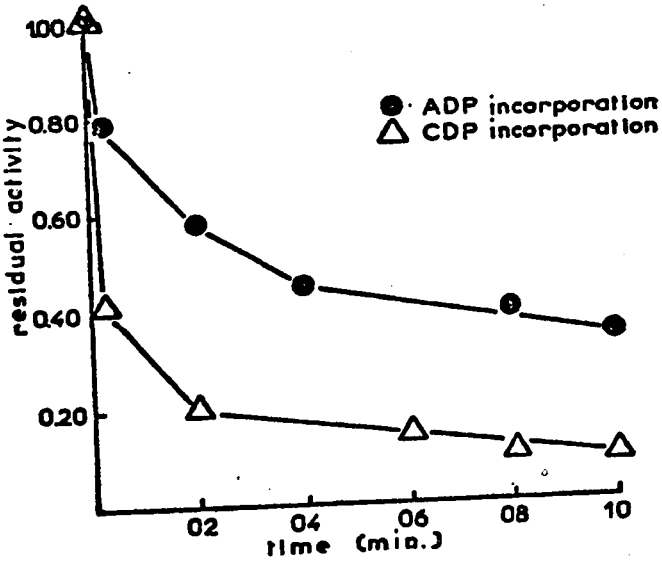


FIG. 13(b). Effect of trypsin-treatment on impure *A. vinelandii* PNPase

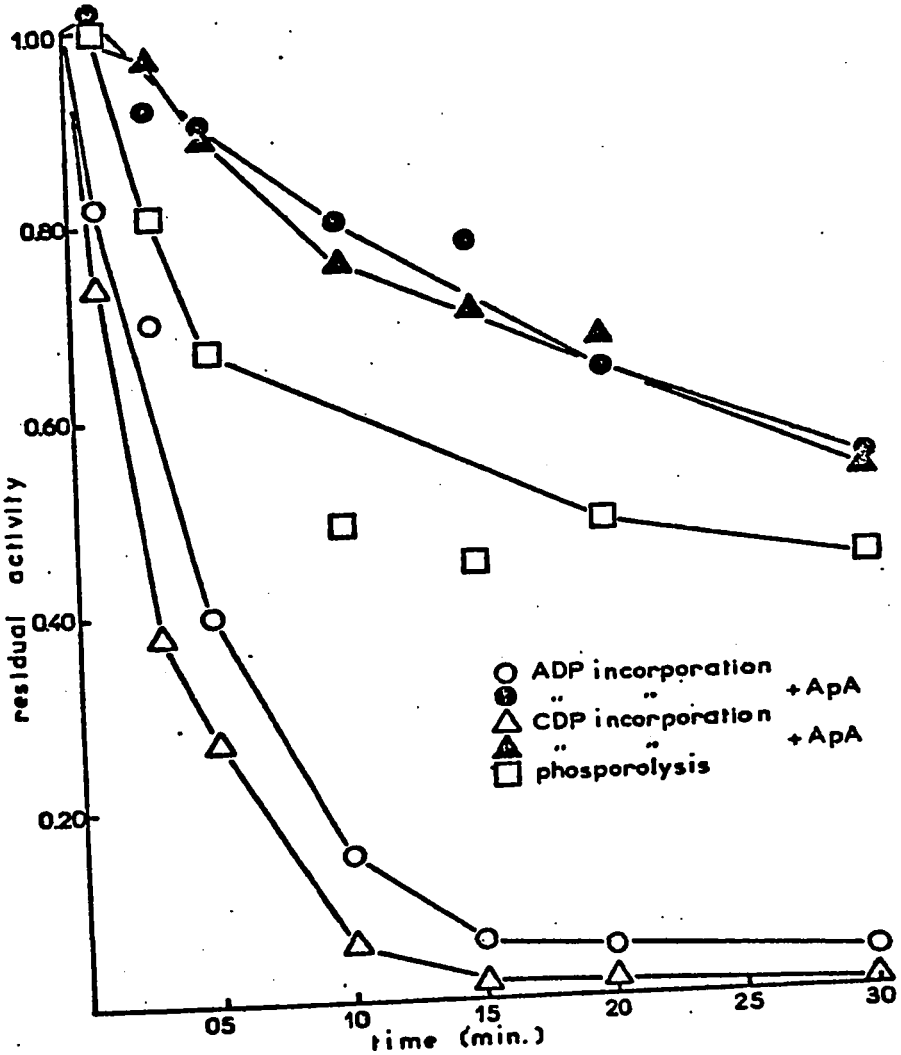


FIG. 14. Effect of trypsin-treatment on activities of pure A. vinelandii PNPase

trypsin ($5x10^{-7}$ M in 1 mM HCl) and mix the two at zero time. At the appropriate times, aliquots were withdrawn, added to an equal volume of soybean trypsin inhibitor ($5x10^{-6}$ M) and kept in ice, until the various activities could be measured. The zero time control was also incubated at 37° for 30 min but the appropriate amount of inhibitor was added before the trypsin. Earlier experiments had shown that the activity of this control was stable under these conditions and did not differ from a control maintained in ice with no additions.

As we can see in Figure 14, the ADP- and CDP-incorporation activities, measured in the absence of ApA, drop precipitously after addition of trypsin and eventually level off at a low value. Other experiments have shown that these values do continue to fall and eventually reach zero. The point to note is that the two curves can be fairly said to be coincident, the difference between the two being very small. The curves for ADP- and CDP- incorporation with ApA, drop much more slowly, although they too tend asymptotically to zero after long incubations. Again the curves are more or less coincident; however, I have not been able to find conditions in which there is residual incorporation activity with ApA, but zero activity without ApA. On the other hand, at very low trypsin concentrations, the activities with ApA remain unchanged for appreciable periods of time, while the activities without ApA drop slowly. This is a matter of

degree, since after long incubations the curves qualitatively resemble those shown. Finally, the enzyme loses phosphorolytic activity at a rate intermediate between the rates for incorporation activity with and without ApA. This activity of course, is unaffected by ApA.

The losses in activity are accompanied by changes in the physical properties of the enzyme (Fig. 15). The experiment was carried out exactly as described above. For the "protein" gels, 100 µl samples of the PNPase-trypsin-inhibitor mixture were mixed with 50 µl 10% sucrose and the whole applied to each gel. Thus each gel contained 8 µg PNPase, 0.25 µg trypsin and 5 µg soybean trypsin inhibitor. For the "activity" gels, 5 µl samples of the mixture were mixed with 100 µl of water and 50 µl of 10% sucrose, and the whole applied to each gel. Thus each gel contained about 2.5 ADP-incorporation units (before trypsin digestion). Electrophoresis was as described in the "Methods" section.

The protein gels all exhibit two bands other than the enzyme band; the slow-moving "anomalous" band, mentioned in another section; and the fast-moving band, which I identified as soybean trypsin inhibitor in another experiment. The amount of trypsin is below the limits of detectability of the staining technique. The middle band is the enzyme band. In the zero time protein gel, the enzyme appears as a heavy band with some leading and trailing diffuseness. In most cases, however, it appears as a single, heavy, sharp

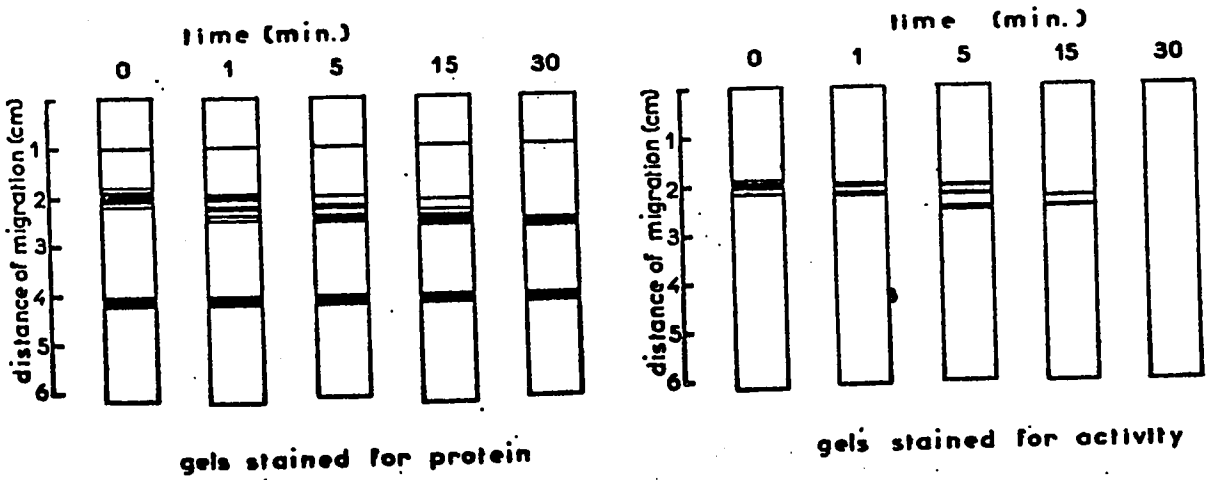


FIG. 15. Effect of trypsin-treatment on electrophoretic mobility of pure A. vinelandii PNPase

band. For the purposes of this discussion, I will call it band "A". When the enzyme is digested with trypsin, after one minute, two more bands appear, a heavy one, band "B" and a light one, band "C", while band "A" becomes sharper and slightly less intense; after 5 min it becomes much weaker, band "B" more intense and band "C" very heavy. After 15 min bands "A" and "B" have almost disappeared, while band "C" has become as heavy as the original pre-digestion band "A". After 30 min, only band "C" remains. These changes are paralleled by the activity gels, except that after 30 min band "C" shows no activity.

The inference here is that trypsin attacks PNPase in two consecutive steps, finally yielding a trypsin-resistant core. However, the core is not completely resistant, since it continues to lose activity. Further, as a purely subjective impression, it seemed in experiments carried through longer time spans that band "C" continued to be displaced towards higher mobility with longer trypsin treatments. Unfortunately, the effect was so slight that I could not detect it consistently by eye, nor by scanning the gels with a densitometer.

Judging from the result in Figures 14 and 15, it is a possibility that the enzyme loses activity after an initial trypsin digestion, simply due to some inherent instability. To eliminate this possibility, I digested a large amount of the enzyme, then removed the trypsin and

inhibitor by DEAE-Sephadex chromatography.

The enzyme used was 30% stimulated by ApA before digestion, but after trypsin treatment was 14-fold stimulated by ApA. By comparison with Figure 14, this corresponds to a digestion time of 10-20 minutes. After purification, the enzyme was 23-fold stimulated by ApA and this value remained the same over a period of a few days. Gel electrophoresis revealed one band (aside from the "anomalous" band), which was faster-moving than the untreated enzyme band, i.e. band "C" of Figure 15. Thus the enzyme "core is stable and the losses of activity mentioned above are caused only by trypsin.

Band "C" does not have an absolute primer requirement. In other gel experiments, activity appeared at band "C" whether or not ApA was included in the gel incubation mixture, although, in the absence of ApA, the band was extremely weak and barely detectable. I could detect no other activity band in the gels.

In every case the results were the same, whether assays were carried out in the presence or absence of BME (Table 24). In the digestion experiments described above, the final BME concentration in the assay medium was 2.5×10^{-5} M which does not appreciably stimulate the oxidized, undigested enzyme (viz. Fig. 16b) and is also much lower than the concentration mentioned in reference 67, in their work with M. luteus PNPase. The experiment was repeated

TABLE 24

Effect of BME on trypsin-treated pure enzyme

Addition to ADP-incorporation assay	Digestion time (min)		
	0	5	10
	residual activity		
none	1.00	0.14	0.06
BME (10 mM)	0.98	0.14	0.06
ApA (100 µg)	1.06	0.33	0.09

TABLE 25

Effect of BME on trypsin-treated inpure enzyme

Addition to ADP-incorporation assay	Digestion time (min)		
	0	5	10
	residual activity		
none	1.00	0.37	0.08
BME (10 mM)	1.10	0.37	0.08
ApA (100 µg)	1.01	0.50	0.09
BME + ApA	1.22	0.47	0.10

with an impure A. vinelandii PNPase (ADP spec. act. 500 units/mg), which had been oxidized, reduced, and dialyzed free of BME, to eliminate the possibility that the presence of BME altered the results (Table 25). Although the digestion is much faster, due to the higher trypsin concentration ($5x10^{-6}$ M), it is obvious that BME alone has no effect in restoring lost activity.

g) Oxidation

The single discovery which made possible the work embodied in this thesis, from the success of the purification, to the study of the properties of polynucleotide phosphorolylase, was that of the reversible oxidation of the enzyme. At the time Dr. Fitt was verifying the preliminary report by Klee and Singer (130), that BME partially reversed the effect of trypsin on M. luteus PNPase, and I was to investigate this phenomenon with respect to the A. vinelandii enzyme. The only available source of the enzyme was a recent preparation which had yielded a seemingly partially denatured polynucleotide phosphorylase, due to the DEAE column having accidentally run dry (Table 26). I found that treatment with trypsin followed by BME resulted in a dramatic increase, rather than a decrease in the activity of the enzyme. This quickly led to the discovery that the enzyme had been reversibly oxidized rather than irreversibly denatured, that BME could reduce the enzyme back to its

TABLE 26

Progressive oxidation of PNPase during purification in absence of BME

Enzyme Fraction	ADP-incorporation (units)		
	during preparation	after two weeks	
	no BME	no BME	with BME
crude extract	20,100	20,600	19,600
Ammonium Sulf.	20,800	13,500	21,700
DEAE-Sephadex	16,400	-	-
G-200	8,100	7,200	21,500

native reduced form, and that in fact the preparation had been successful.

i) Oxidation during purification

I shall now present the evidence that the enzyme is reversibly oxidized and that the group undergoing oxidation is a sulfhydryl group. The effect of BME was tested on samples saved from various stages of the purification (Table 26). Note that two weeks had elapsed from the completion of the G-200 step and four weeks from the preparation of the crude extract. Although the crude extract had not undergone any oxidation, the AS fraction was stimulated by BME, yielding the required activity. (No sample of the DEAE eluate was available.) After the G-200 step, the enzyme was greatly stimulated by BME, again yielding the required activity. On the basis of these results, it is not possible to determine conclusively whether the oxidation took place on the DEAE column, or at some later stage in the preparation. Even without exposure to the drastic conditions of a dry DEAE-Sephadex column, the AS fraction lost activity over a period of four weeks, and the G-200 fraction was even further oxidized during a two-week interval. Such losses of activity had been noted before, but were attributed to a non-specific irreversible denaturation. Finally, the losses of activity shown here were not reversed by ApA, nor were they attributable to the

pH of medium (pH 7.4) since these latter losses appear to be associated with very pure enzyme preparations as discussed elsewhere.

ii) Reversal of oxidation

For regeneration of the oxidized enzyme, the reducing agent of choice is β-mercaptoethanol (BME), on account of its complete solubility in water and its low cost. (Disadvantages are its volatility and foul odour.) The stimulation or regeneration of the oxidized enzyme by BME can be quite dramatic (Fig. 16a). The enzyme used had been oxidized on a DEAE-Sephadex column as discussed later. The ADP-incorporation activity was stimulated by increasing BME concentrations in the assay with a maximum stimulation of six-fold at 20 mM BME. At higher concentrations BME inhibited the activity. Similarly, the phosphorolytic activity was stimulated by BME in the assay; however, BME stimulated this activity even at concentrations of 200 mM, which inhibited the ADP-incorporation activity. Moreover, the maximal stimulation was much less than that for the ADP activity.

This stimulation of the various activities was observed even in enzyme preparations which were only slightly oxidized (Fig. 16b). In this case the pure, reduced enzyme had been stored in TEB 8.2 buffer, but had nevertheless developed a slight BME requirement. 1 mM BME

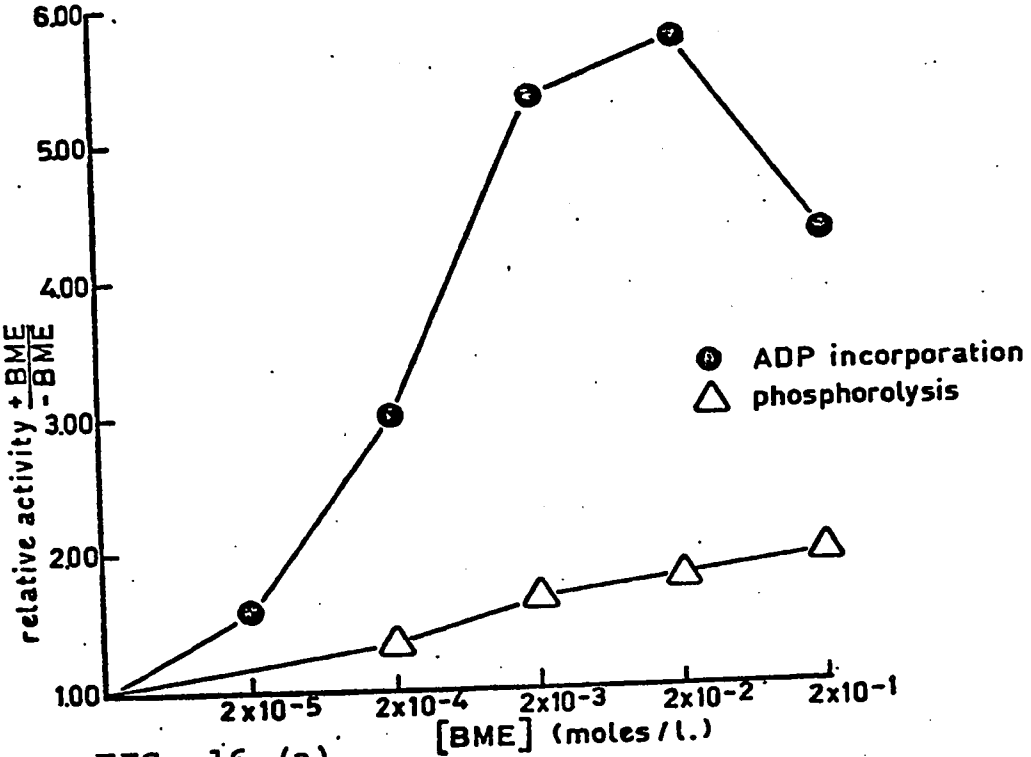


FIG. 16 (a).

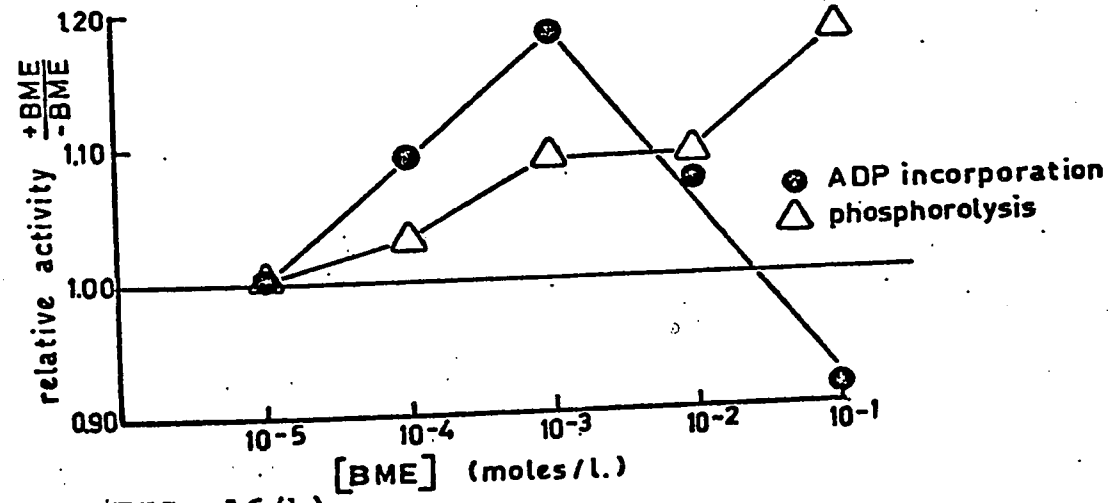


FIG. 16 (b)

FIG. 16. Stimulation by BME of oxidized PNPases
a) highly oxidized
b) slightly oxidized

appeared to be the optimum concentration in the ADP-incorporation assay, and higher concentrations inhibit. The phosphorolytic activity, on the other hand, is increasingly stimulated by higher BME concentrations.

I chose to use 10 mM BME as the standard concentration in the assay, because it is effective in fully regenerating the oxidized enzyme, and does not significantly inhibit the fully-reduced enzyme. The same BME concentration was used in the phosphorolytic assay, because it seemed that at the higher concentrations, I was no longer dealing with a reversal of oxidation, but rather with a simple stimulation.

Other sulfhydryl reagents are as effective on BME in regenerating the oxidized enzyme. For instance, thioglycerol was equivalent to BME, glutathione (GSH) was very slightly less effective, and 1,3-dimercaptopropanol was slightly more effective in stimulating the oxidized enzyme in the assay. Dithiothreitol (Cleland's reagent) was also effective in stimulating the assay, but storage of the oxidized enzyme in 10 mM dithiothreitol produced a faint green precipitate and inhibition of the activity, on the one occasion it was employed. All these reagents therefore did not seem to have any marked advantage over BME, so I continued to use the latter in all my work.

iii) Slow air-oxidation

On the basis of the discovery of this reversible oxidation I developed an effective purification procedure for A. vinelandii PNPase. I was able to obtain an enzyme of at least 95% purity, as outlined previously in this discussion. I then returned to the problem of the postulated oxidation to study it in more detail, under controlled conditions. It was not immediately clear what had caused the enzyme to lose activity. The most obvious hypothesis was that the oxidation was caused simply by oxygen in the air and that storage of the enzyme in the absence of reducing agents could cause an observable, controlled oxidation.

This indeed proved to be the case as we can see in Figure 17. The procedure was simply to place a portion of the enzyme preparation in an open dialysis bag, which had been thoroughly cleaned with NaOH, EDTA and distilled water, and to dialyze against continuous changes of TE 7.4 buffer.

ADP-incorporation activity with and without BME in the assay was monitored as was the sulfhydryl concentration by the method of Boyer (21). Following the curve for ADP-incorporation without BME, during the first few days this remains fairly constant, but then the enzyme starts to lose activity slowly during the extended dialysis, and after a few weeks the residual activity is 58% of the original value.

However, a large fraction of this activity can be recovered by adding BME to the assay mixture. This would

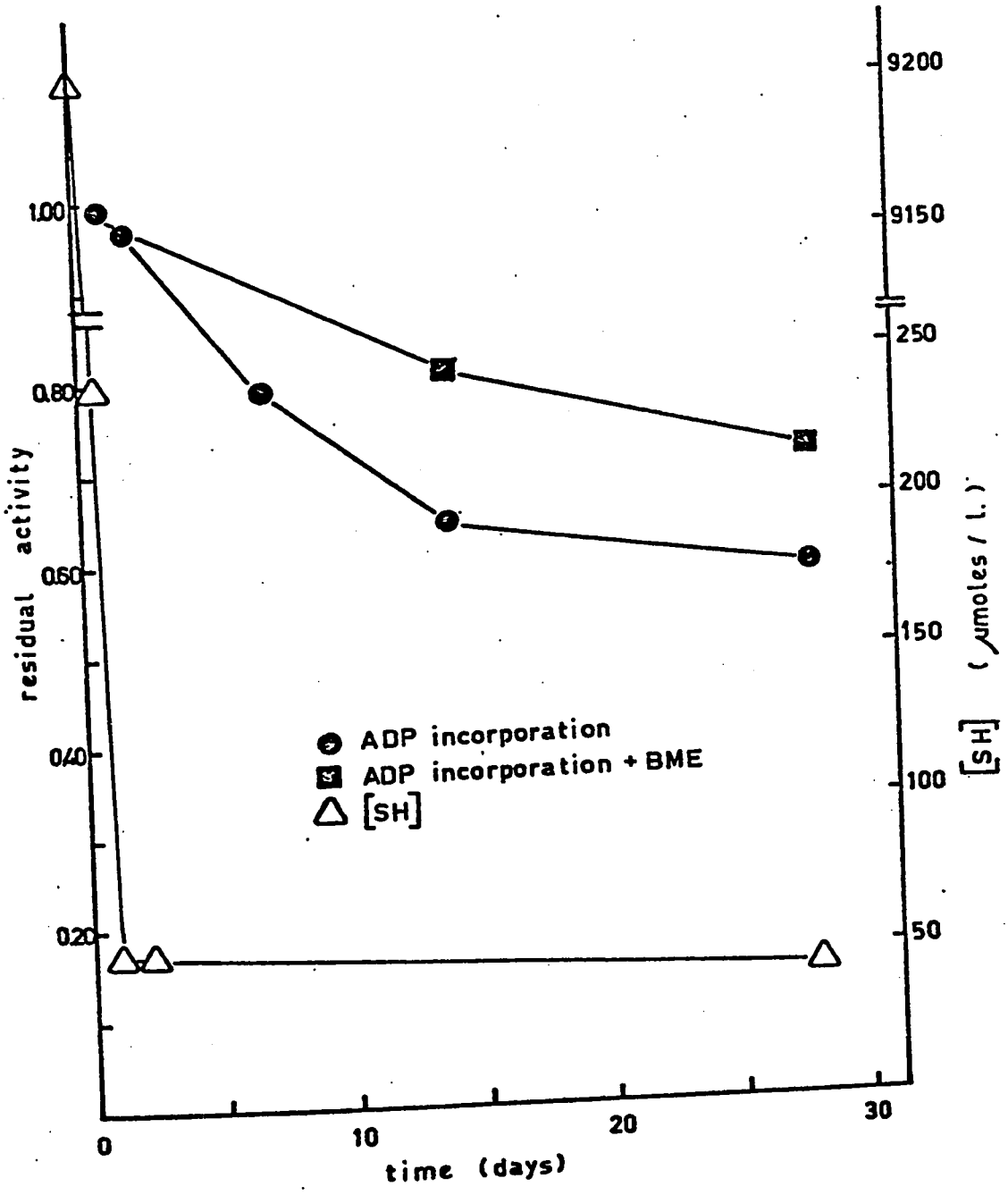


FIG. 17. Slow air oxidation of PNPase

again suggest a reversible oxidation, and since the method is so gentle, it would be hard to attribute the effect to anything except the action of atmospheric O_2. As far as the non-reversible loss of activity is concerned, this would be non-specific denaturation of the enzyme, since as I have shown previously the pure enzyme is somewhat unstable in dilute solutions at pH 7.4. The ApA stimulation was not monitored during this experiment, so at least part of this "irreversible" loss may, in fact, be an increase in primer requirement.

Of particular interest in this figure is the curve for "SH concentration." In the first twenty-four hours, as the dialysis medium was constantly changed, the concentration of sulfhydryl groups (measured by the method of Boyer) dropped rapidly as the BME was removed. After the initial quick drop in concentration, there was a further, very slow loss of sulfhydryl groups, but during the course of the experiment the level never reached zero. During the initial fast drop there was no change in any of the properties of the enzyme. However, during the ensuing slow loss, the enzyme lost activity, which loss was partially reversed by BME in the assay. The inference here, of course, is that sulfhydryl groups on the enzyme are in some way involved in the activity of the enzyme.

In later experiments the slow air-oxidation was not as pronounced, although qualitatively similar. By that time,

all my usual buffers contained large amounts of BME, and all the cold rooms and refrigerators at my disposal were naturally contaminated with BME vapour. It is therefore possible that any attempt to remove traces of BME and induce oxidation were thwarted by the reducing atmosphere of the environment. Nevertheless, I consistently find that enzyme solutions in which the BME concentration is below 1 mM loses activity over a period of time (Fig. 16b), which loss is reversed by the addition of BME to the solution.

Further, the method is not very promising because of the length of time required for any significant changes to become apparent, and the problem is further complicated by increases in primer requirement and losses of activity due to non-specific denaturation. I attempted to speed up the oxidation by bubbling O_2 through a solution of the enzyme, by storing the enzyme under hyperbaric O_2 and by adding oxidizing agents, such as NAD^+, potassium ferricyanide, and p-benzoquinone. With the exception of the last two reagents (about which more later) all these methods caused more irreversible denaturation than reversible oxidation, and were not pursued further.

iv) Fast oxidation on columns

I investigated further the oxidation by air of the enzyme adsorbed to DEAE-Sephadex columns. This study was carried out with pure PNPase. The enzyme in 0.1 M NaCl and

TEB 8.2 buffer was adsorbed to a small column (less than 1 ml column volume) of DEAE-Sephadex, washed thoroughly with TE 8.2 buffer, air was sucked through the column for approximately six hours, and the enzyme was eluted with a large volume of TE 8.2 buffer. Finally, two samples of the column eluate were dialyzed free of NaCl, one against TE 8.2 buffer, the other against TEB 8.2 buffer. In early work with this method I found that it was satisfactory in giving a highly oxidized enzyme (up to six-fold stimulated by BME), but there were problems with eluting the enzyme from the column, yields being of the order of 50%.

It seems that PNPase is very strongly bound to DEAE-Sephadex, and although shallow salt gradients are effective in eluting it, batchwise elutions with high salt concentrations are inefficient, and with the small columns used in these experiments, very large volumes (40-50 times the column volume) are needed to completely elute the enzyme. In fact, I was finally able to elute 100% of the enzyme units applied to the column, measured in the presence of BME. Since the enzyme used was very pure, there was no problem of contamination by other proteins (and of course no additional purification), but this observation explains why large-scale batchwise elutions of the enzyme during early preparations did not give very dramatic purification.

Results from one such column oxidation are shown in Table 27. All results are based on the activity of the

TABLE 27.

Effect of column oxidation on activities of pure PNPase

Enzyme Fraction	Relative ADP-incorporation activity addition to assay		relative phosphorolytic activity addition to assay	
	None	10 mM BME	None	10 mM BME
untreated	0.89	1.00	0.98	1.00
control TE 8.2	0.73	0.92	0.96	1.02
control TEB 8.2	0.99	0.86	1.03	1.05
oxidized TE 8.2	0.32	0.85	0.85	1.05
oxidized TEB 8.2	0.86	0.88	0.94	1.03

untreated enzyme assayed in the presence of 10 mM BME as 1.00. The first point to note is that recovery of phosphorolysis units from the column in the presence of BME is very nearly 100%. No direct method of estimating recovery is available, since the protein concentration in the column eluates is around 0.005 mg/ml. This would give an A_{280} of approximately 0.015, which is too low for accuracy, especially in the presence of BME. Other methods of protein assays are also not sufficiently sensitive. The result for the control enzyme dialyzed against TE 8.2 buffer shows that some oxidation occurred simply due to the dialysis, i.e. the enzyme was some 26% stimulated by addition of BME to the assay medium. (C.f. the untreated enzyme, 11% stimulation.) The enzyme that was oxidized on the column and dialyzed against TE 8.2 buffer was much more extensively oxidized, and was 166% stimulated by BME. The extra oxidation was obviously due to the air sucked through the column. In either case the oxidation was reversible since assaying with BME, or dialysis against BME buffer gave an enzyme with the same properties as the untreated enzyme. A further point is that the phosphorolysis activity was much less affected by oxidation than was the ADP-incorporation activity. Even in the case of oxidized, TEB 8.2 dialyzed enzyme, BME stimulation was only 20% for phosphorolysis.

The oxidation is not reversed by ApA (Table 28). This experiment was carried out as described, except that

TABLE 28

Effect of column oxidation on primer-stimulated PNPase
(all values relative to assay without additives)

Enzyme Fraction	ADP-incorporation activity additions to assay		CDP-incorporation activity additions to assay		Phosphorolytic activity additions to assay
	100 mM BME	100 µg ApA	100 mM BME	100 µg ApA	100 mM BME
control TE 8.2	1.56	1.12	2.04	1.27	1.37
control TEB 8.2	1.00	1.13	0.82	1.45	1.19
oxidized TE 8.2	2.52	1.07	6.20	1.33	1.50
oxidized TEB 8.2	0.87	1.15	0.84	1.46	1.18

the enzyme used was 95% pure and was stimulated 10% by ApA in the ADP-incorporation assay. The results are expressed relative to the assay with no additions, rather than relative to the untreated enzyme, because recoveries from the columns were only 50-70% for this experiment. Judging from the results for ADP-incorporation activity, the enzyme was extensively oxidized simply by dialyzing against TE 8.2 buffer. The column-oxidized, TEB 8.2-dialyzed enzyme was, however, very much more oxidized. In each case, dialyzing against TEB 8.2 buffer gave an enzyme with no BME requirement. In no case did ApA reverse the oxidation; in fact, there was some suggestion that the oxidized enzyme was less stimulated by ApA than was the reduced enzyme.

The CDP-incorporation activity is much more sensitive to oxidation. Simple dialysis gave a two-fold stimulated enzyme, and the column oxidation gave a six-fold stimulated enzyme. Again the oxidized enzyme appeared to be less stimulated by ApA than the reduced enzyme, and ApA did not reverse the oxidation.

An interesting further point is that the high BME concentration in the assay (100 mM) inhibited the CDP activity of the reduced enzyme more than it did the ADP activity (c.f. Fig. 16).

The air-oxidation was studied in more detail with a pure PNPase preparation. The oxidation procedure was the same as above, but rather than simply assaying for activity

by the normal procedure using 10 min incubations, ADP-incorporation was determined as a function of time. (Fig. 18). In the figure, "O" refers to the oxidized enzyme dialyzed against TE 8.2 buffer, and "OB" refers to the oxidized enzyme dialyzed against TEB 8.2 buffer. In each case, the curves drawn are the best "least square" fit for the points between 3 and 10 min. Enzyme "OB" (which was, of course, the same as the untreated native enzyme) was not stimulated by BME, with or without ApA in the assay. The lag phase, if any, was certainly less than one minute, which is expected for an enzyme not stimulated by ApA in the normal assay. In the case of enzyme "O", BME stimulated the rate of reaction, but did not eliminate a lag phase of some two minutes. This would suggest that the transformation from an oxidized to a full-reduced state takes about one minute. A repetition of these results suggested that the reduction time may be even shorter but a very short lag phase still persists with the oxidized enzyme. Further, the length of the lag phase does not appear to be dependent on the BME concentration. A summary of these experiments is shown in Table 29. The important point is that BME, whether by dialysis or in the assay, completely reverses the oxidation.

Similarly, for the phosphorolytic activity, enzyme "O" is appreciably stimulated by 5 mM BME, whereas enzyme "OB" is only slightly stimulated (Fig. 19). In this case there is no lag phase, which is good evidence that the lag

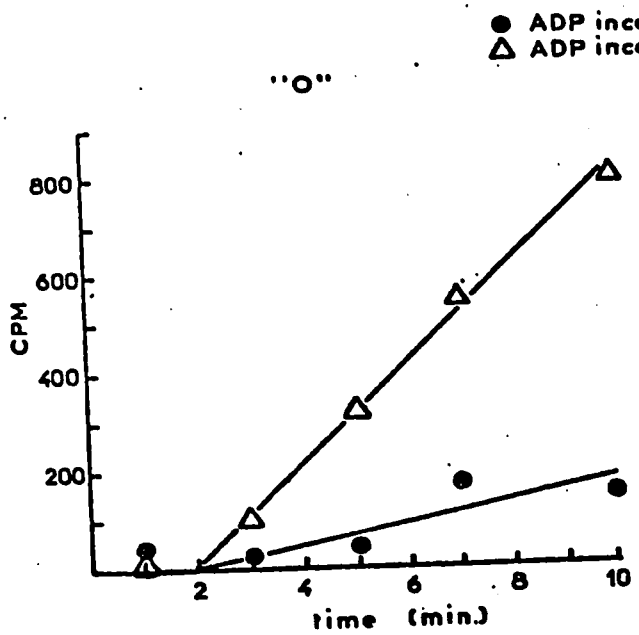


FIG. 18(a)

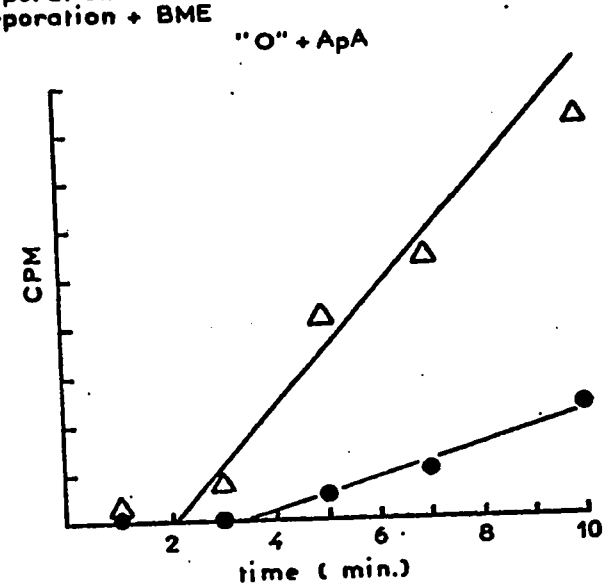


FIG. 18(b)

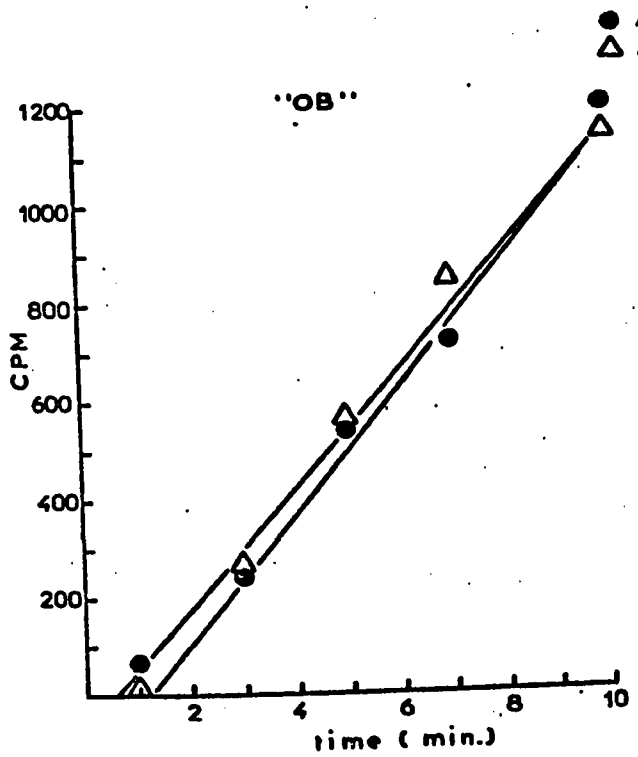


FIG. 18(c)

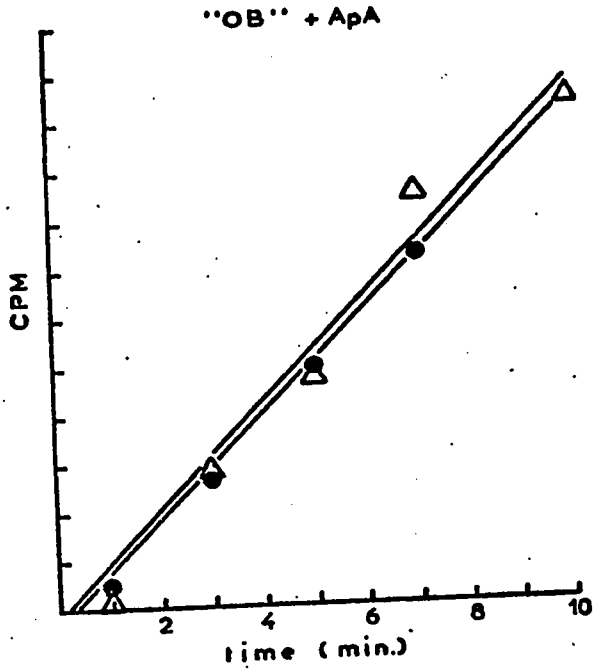


FIG. 18(d)

FIG. 18. Effect of oxidation on time course of incorporation activity

a) oxidized enzyme
b) oxidized enzyme assayed with ApA
c) oxidized and reduced enzyme
d) oxidized and reduced enzyme assayed with ApA

TABLE 29

Inhibition of ADP-incorporation and lag phase caused by oxidation

Enzyme Fraction	ApA in assay	[BME] in assay (mM)			
		0		5	
		slope (cpm/min)	intercept or lag phase (min)	slope (cpm/min)	intercept or lag phase (min)
"O"	-	22	1.90	101	1.90
"O"	100 µg	33	3.30	118	2.05
"OB"	-	133	0.95	124	0.65
"OB"	100 µg	112	0.40	114	0.60

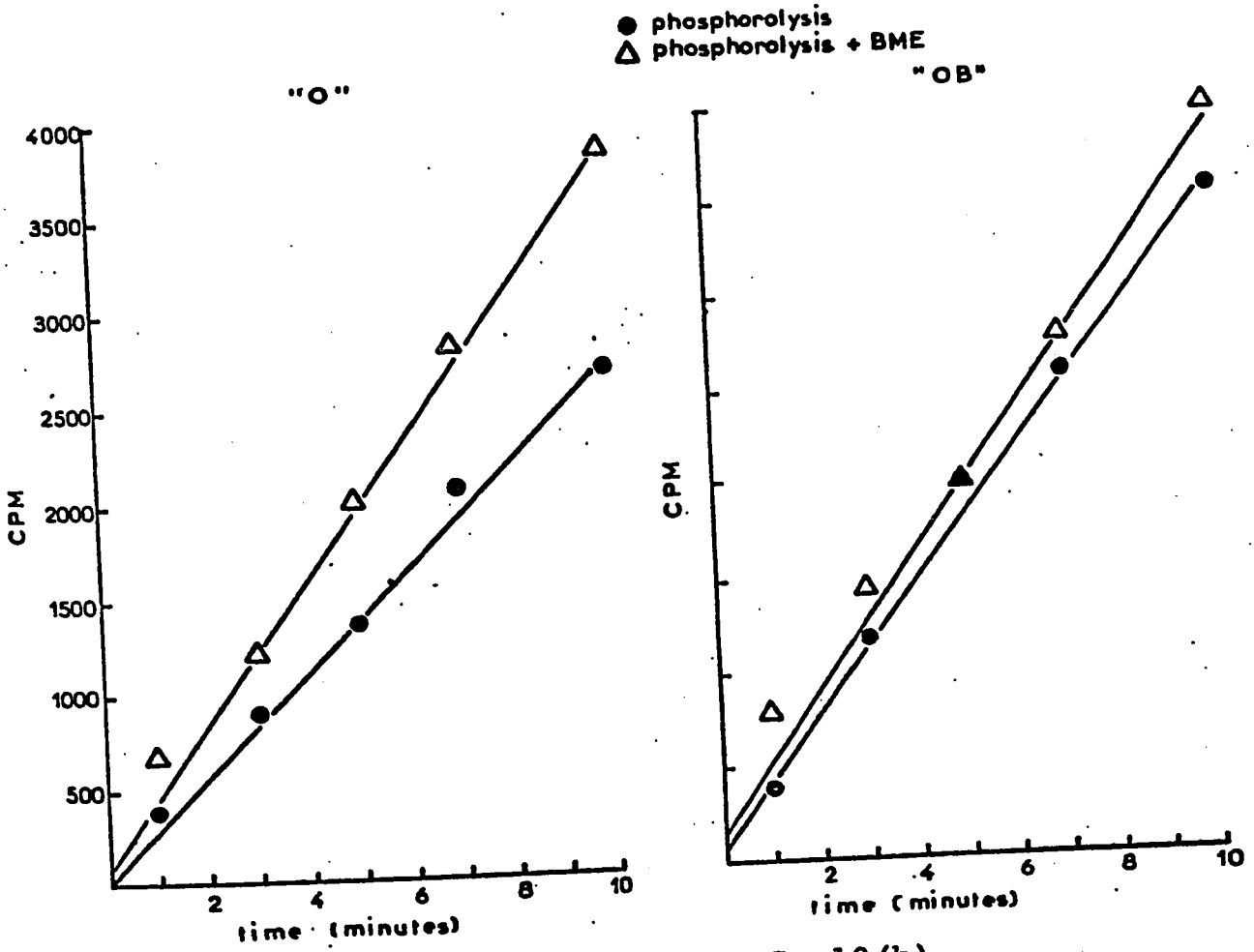


FIG. 19 (a)

FIG. 19 (b)

FIG. 19. Effect of oxidation on time-course of phosphorolysis activity

(a) oxidized enzyme
(b) oxidized and reduced enzyme

phase in incorporation activity is genuine, and not an artefact of the incubation procedure. In this oxidation experiment enzymes "O" and "OB" were tested for phosphorolytic activity at various BME concentrations (Table 30). The values in the table are the slopes taken from graphs of which Fig. 19 is one example. It seems clear that BME regenerated or stimulated both the oxidized and reduced enzymes to the same extent.

Another very important change in the enzyme, caused by oxidation, is in the ADP-Mg^{++} requirement (Fig. 20). This experiment is similar to that shown in Figure 3, except that a pure enzyme, stimulated five-fold by BME, was used. The result is quite striking, in that the optimum ADP/Mg^{++} ratio is 2 for the oxidized enzyme rather than 4, as for the reduced enzyme. Other than the changes discussed above, the oxidized and reduced enzymes are indistinguishable. They have the same pH optimum. The two forms are not separated on polyacrylamide gel electrophoresis, appearing in each case as the same single band of protein and activity, both in the presence and absence of BME.

h) Oxidizing reagents

To identify the groups involved in the oxidation, I carried out a series of experiments on the enzyme using various oxidizing and sulfhydryl-specific reagents.

TABLE 30

Stimulation of phosphorolytic activity of oxidized PNPase by BME

Enzyme Fraction	[BME] in assay (mM)			
	0	1	10	100
"O"	0.58	0.78	0.80	0.91
"OB"	0.77	0.81	0.79	1.00

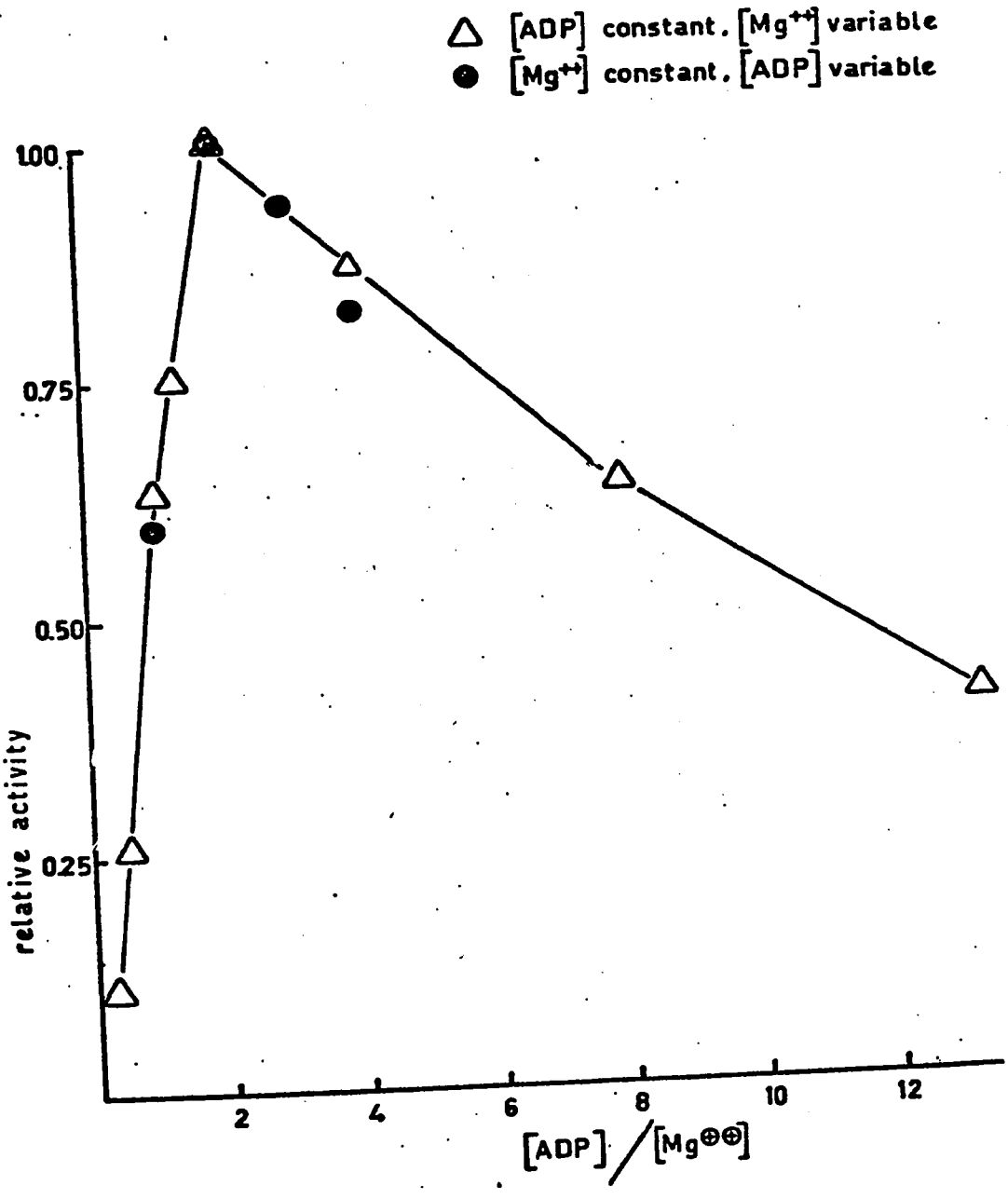


FIG. 20. ADP and Mg^{++} requirement for oxidized PNPase

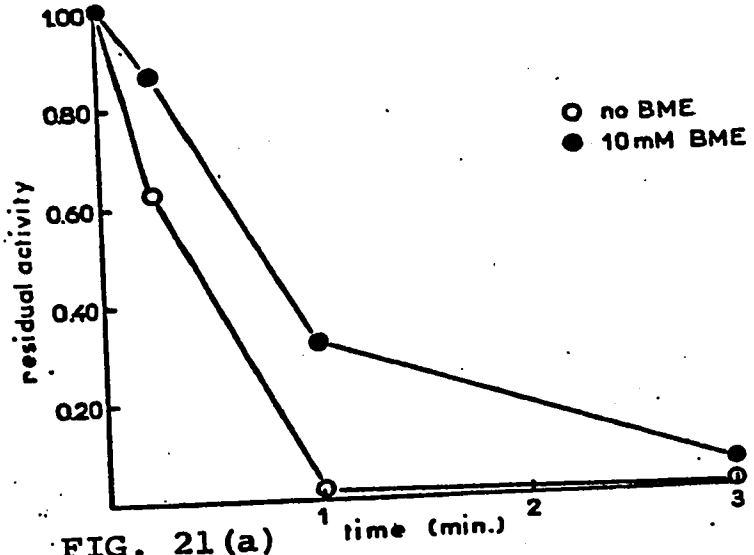


FIG. 21(a)

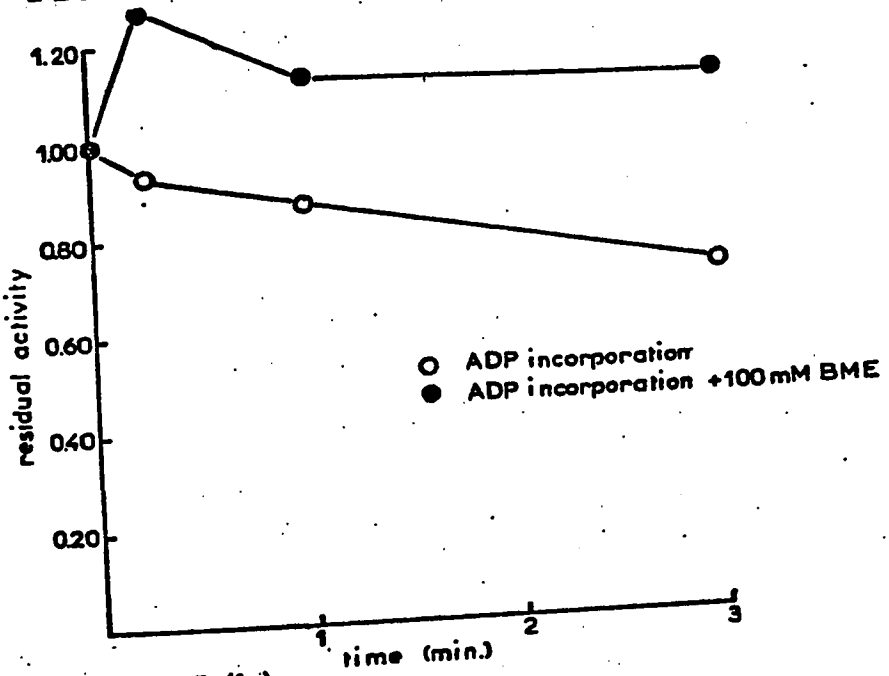


FIG. 21(b)

FIG. 21(a). Effect of GSSG on highly-purified PNPase

FIG. 21(b). Effect of ferricyanide on highly-purified PNPase

i) GSSG

I had found that BME was effective in reversing aerial oxidation, and that glutathione (GSH) also stimulated the activity of the air-oxidized enzyme. Since I did not have the oxidized dimer of BME, I treated the enzyme with oxidized glutathione (GSSG). Results are shown in Figure 21a. The fully reduced enzyme in TEB 7.4 buffer was mixed with an equal amount of 10 mM GSSG, and incubated at 37°. Samples were withdrawn at the indicated times and assayed for ADP-incorporation activity, with and without 10 mM BME. (Note that the GSSG concentration in the assay was 1 mM.) The zero time tube contained no GSSG. As we can see from the figure, GSSG caused a partially reversible "oxidation" of the enzyme but this was accompanied by severe irreversible losses of activity. This was not due simply to the presence of GSSG. A sample of the enzyme was incubated 3 min at 37° with 5 mM GSSG and then dialyzed overnight against a large volume of TE 7.4 buffer. After this treatment the enzyme was stimulated three-fold by 10 mM BME in the assay but the recovery of units even with BME was only 5.3%.

ii) Potassium ferricyanide

Next I tested the effect of potassium ferricyanide (PF) (Fig. 21b). In this case the enzyme in TEB 7.4 buffer was mixed with an equal volume of 2 mM PF, incubated at 37° and samples withdrawn at the indicated times. These were

assayed for ADP-incorporation activity, with and without 100 mM BME. The stimulation of the activity by PF and BME together was not observed on other occasions. PF alone caused gentle reversible oxidation. In fact, when the incubation time is extended to 2 h, the enzyme retains 81% of the original activity with BME, while only 47% remains without BME. This result is qualitatively reproducible in a variety of conditions.

With a pure enzyme at pH 9.0 and a 37° incubation, PF gave the result shown in Fig. 22a. (Note that the concentration of ferricyanide in the 10 min assay subsequent to the incubation was one-fifth the concentration in the incubation.) PF caused a dramatic increase in BME requirement, but there was obviously some irreversible loss of activity. The loss of activity was faster at high temperatures than at low ones (Fig. 22b). In this case the ferricyanide concentration was 100 mM, and the low temperature "incubation" tube was merely stored in ice. Assays were performed without BME. The effect is, of course, also dependent on concentration of ferricyanide (Fig. 23a). In this case incubation time was 4 h at 37°. It is clear that at no concentration was there a simple oxidation, with no irreversible denaturation. Further, only reducing agents are effective in reversing the oxidation. Although this enzyme was 10% stimulated by ApA in the native state, the oxidized form was, in fact, slightly inhibited by ApA. This is

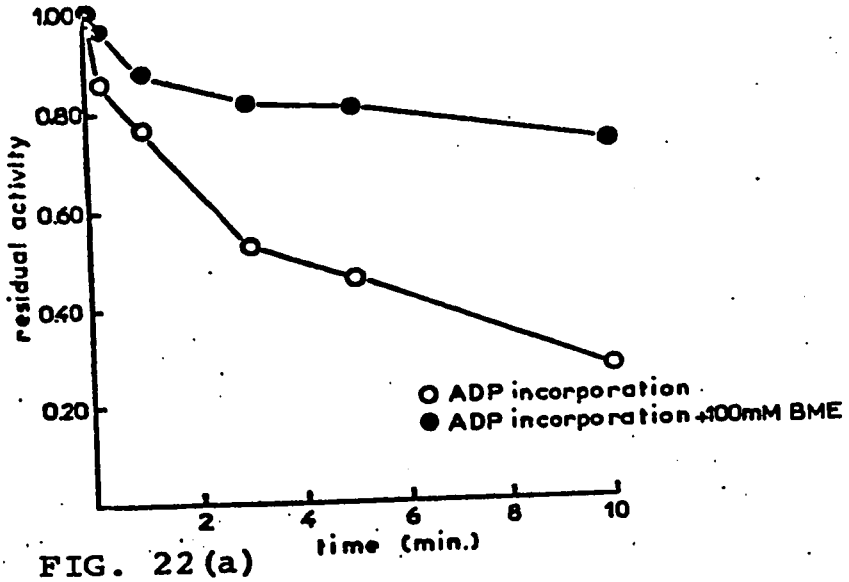


FIG. 22(a)

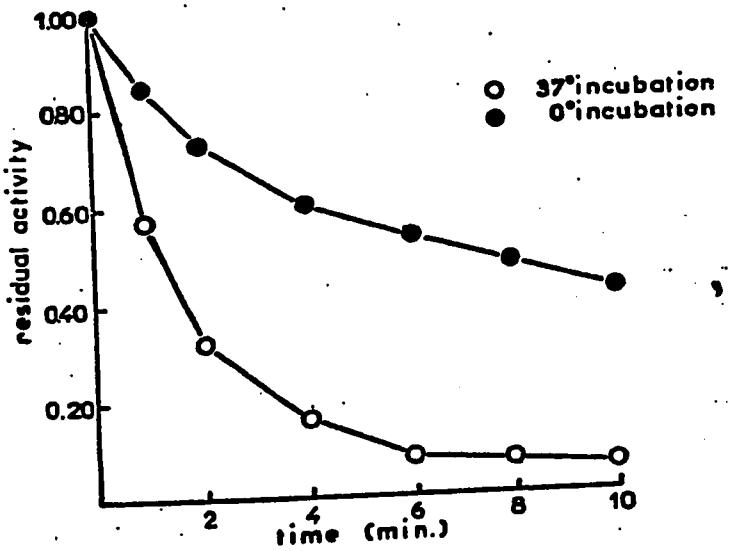


FIG. 22(b)

FIG. 22(a). Effect of ferricyanide on pure PNPase

FIG. 22(b). Effect of temperature of ferricyanide-treatment on pure PNPase

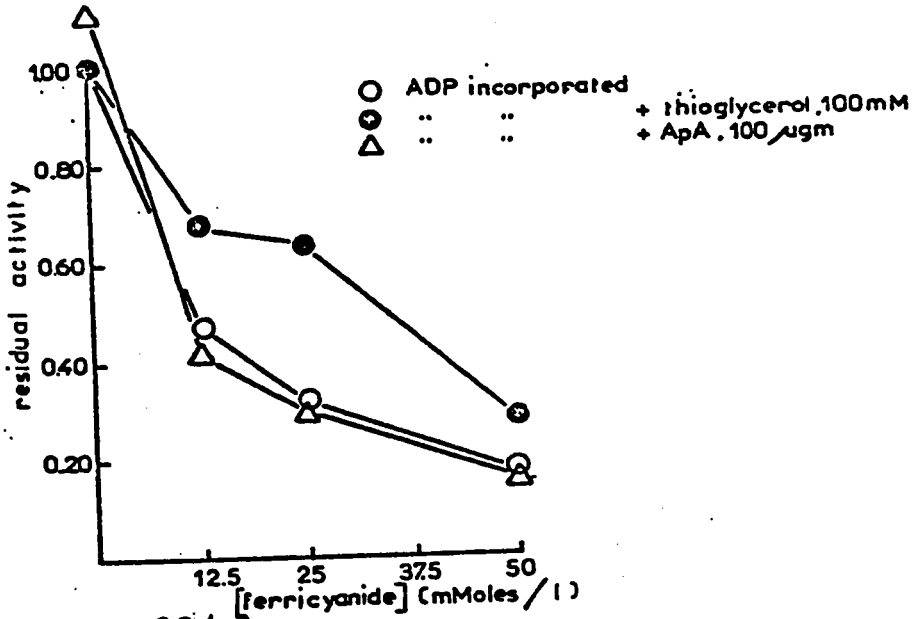


FIG. 23(a)

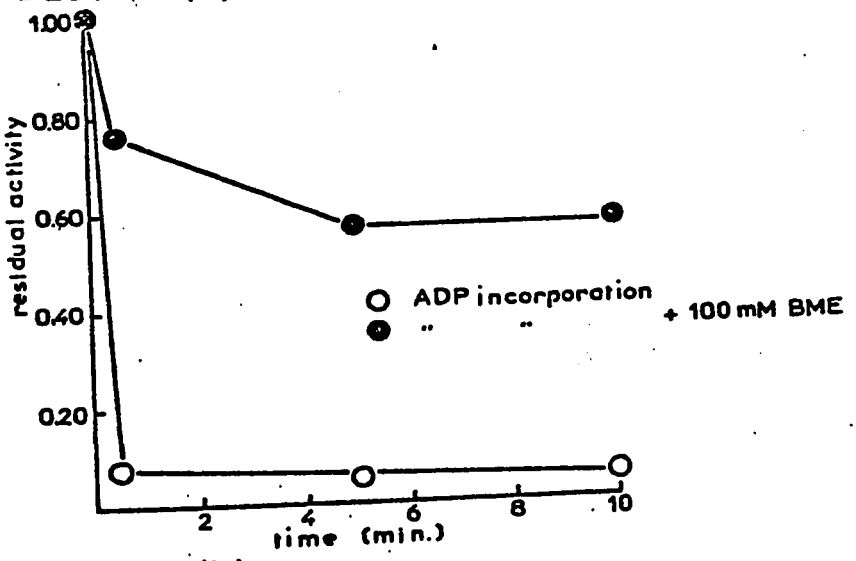


FIG. 23(b)

FIG. 23(a). Effect of concentration of ferricyanide on pure PNPase

FIG. 23(b). Effect of p-benzoquinone on highly-purified PNPase

analogous to the effect observed for the air-oxidized enzyme (Table 28). The loss of activity was not simply due to inhibition by ferricyanide. The enzyme was treated with 0.07 M PF at 37° for 10 min and found to be two-fold stimulated by BME with 10% recovery of activity. After dialysis against several changes of TE 9.0 buffer for 3 days, the treated enzyme was 3.3-fold stimulated by BME with 43% recovery of activity. Thus the observed oxidation was caused by PF, rather than by air. Further, 500 mM potassium ferrocyanide did not inhibit PNPase, so the effect of ferricyanide is oxidation rather than non-specific inhibition.

Treatment with ferricyanide alters the electrophoretic properties of the enzyme. The ferricyanide-treated enzyme, dialyzed free of ferricyanide, had a "smeared" pattern on polyacrylamide gels with no distinct protein bands. The activity gels also had this indistinct area of staining, which was faster moving than the native enzyme. The result was the same whether or not the "oxidized" enzyme was pre-incubated with BME.

iii) p-benzoquinone

The oxidation of the enzyme by p-benzoquinone was studied (Fig. 23b). This experiment was carried out exactly as that shown in Figure 21b, substituting p-benzoquinone for potassium ferricyanide. The reaction is so fast that it

appears to be an instantaneous inhibition rather than a progressive oxidation; nevertheless, the effect is partially reversed by BME. When the pure enzyme in TEB 8.2 was treated with 2 mM p-benzoquinone, the residual activity was 58%, but 100% with 10 mM BME (Table 31). The treated enzyme was divided into two portions: one, dialyzed against TEB 8.2 buffer ("QB"), retained 74% of the original ADP activity, and 92% of the phosphorolytic activity; the other, dialyzed against TEB 8.2 buffer ("Q"), retained only 52% of the original ADP activity, was stimulated two-fold by 10 mM BME, and retained 65% of its phosphorolytic activity. The treated and dialyzed fractions, however, had a light-brown colour, unlike that of the native enzyme, and had severely altered electophoretic patterns, roughly similar to the ferricyanide-treated enzyme; therefore, side reactions other than the oxidation had occurred. I should mention that 20 mM tetra-hydroxyquinone had no effect whatever on the enzymic activities.

Finally, I tried NAD^+ as an oxidizing agent. 10 mM NAD^+ in the assay caused 22% inhibition and even with the addition of 100 mM BME to the assay, the inhibition was still 20%. Thus none of the chemical oxidizing agents tried so far have been suitable for the study in controlled conditions of the reversible oxidation, since they all either inhibited the enzyme irreversibly, or caused irreversible structural changes in the enzyme protein.

TABLE 31

Effect of p-benzoquinone and dialysis on pure PNPase
(see text for explanation)

Enzyme Fraction	Assay	[BME] in assay	Relative Activity		
			before treatment	after treatment	after treatment and dialysis
"Q"	ADP-incorp.	-	-	0.58	0.23
"Q"	"	10 mM	1.00	1.00	0.52
"QB"	"	-	-	-	0.74
"QB"	"	10 mM	-	-	0.74
"Q"	phosphorolysis	-	-	0.97	0.56
"Q"	"	10 mM	1.00	1.13	0.65
"QB"	"	-	-	-	0.87
"QB"	"	10 mM	-	-	0.92

iv) p-chloromercuribenzoate

Definitive results were obtained with sulfhydryl-specific reagents. The most obvious one is p-chloromercuribenzoate (PMB) which is considered to bind free sulfhydryl groups specifically and reversibly, and, in fact, forms the basis of the Boyer method for assaying sulfhydryl groups (c.f. Fig. 17).

A 95% pure PNPase in TEB 7.4 was treated with varying amounts of PMB in ice for 15 min, then assayed for ADP-incorporation with and without 100 mM BME. (Fig. 24a.) The PMB concentrations are those in the assay, and are uncorrected for any reaction with the BME present in the enzyme preparation (0.2 mM). Judging from the results in the figure it seems that both a reversible and an irreversible reaction occurred.

The reaction between PNPase and PMB is not instantaneous. (Fig. 24b.) The enzyme in TEB 7.4 was kept in ice with 222 μM PMB, samples withdrawn at the indicated times and assayed, with and without BME. The reaction was almost completely reversible and was complete in less than one hour. There is some indication that a non-reversible process continued after this time. It is, however, clear that BME was effective in reversing the reaction during the assay time at these PMB concentrations.

These results could be repeated with the homogeneous enzyme in TEB 8.2. (Fig. 25.) This enzyme was stimulated

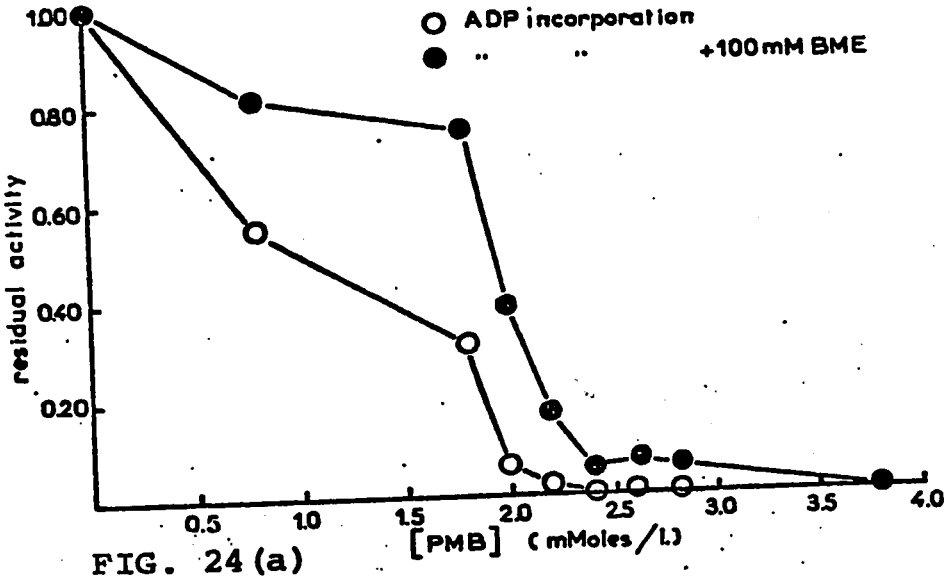


FIG. 24 (a)

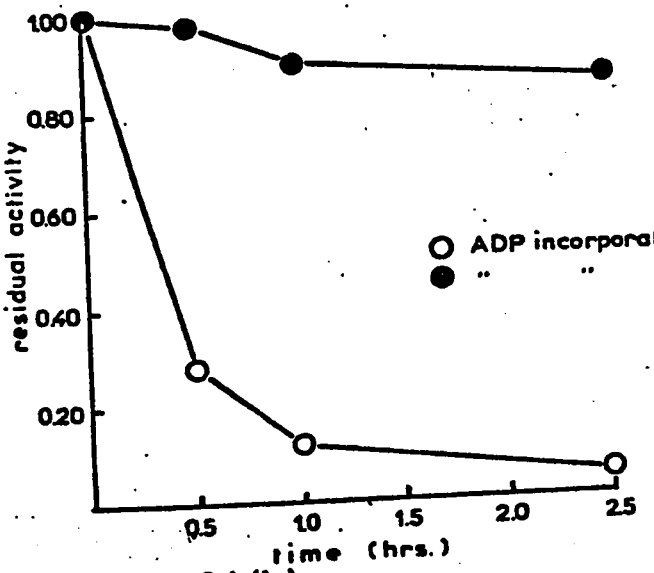


FIG. 24 (b)

FIG. 24(a). Effect of PMB on highly-purified PNPase

FIG. 24(b) Time-course of inactivation of PNPase by PMB

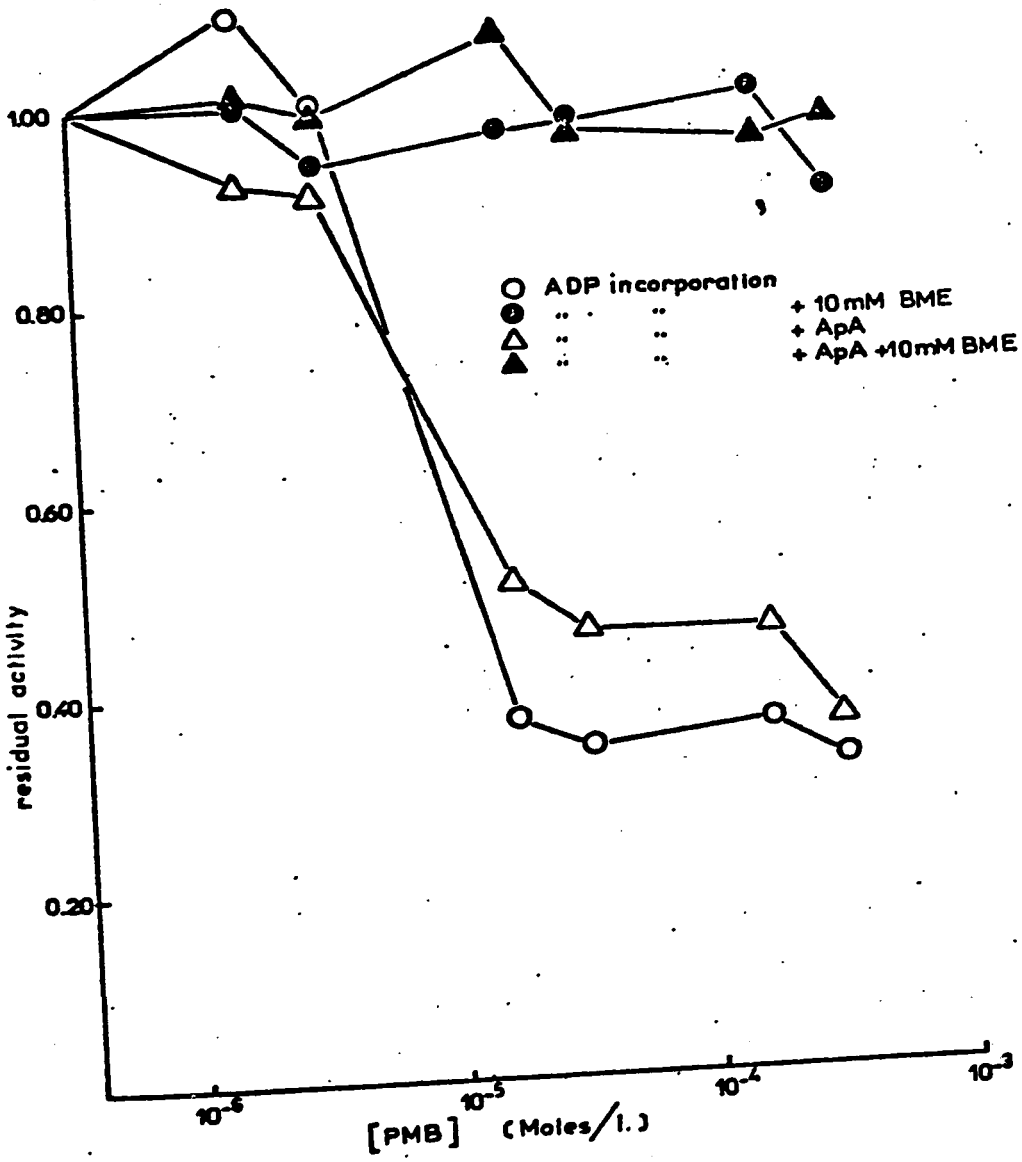


FIG. 25. Effect of PMB on pure PNPase

neither by BME nor by ApA. The procedure was to store samples of the enzyme with varying concentrations of PMB for one hour in ice, then assaying for ADP-incorporation, with and without 10 mM BME, in each case with and without 100 µg ApA. The PMB concentrations in the figure refer to those during the one-hour treatment; during the subsequent 10 min assay the PMB concentrations were one-fifth of those values. Note that the abscissa is logarithmic.

The result is more dramatic than that in Figure 24a. At some threshold PMB concentration between 3.3×10^{-6}M and 1.7×10^{-5}M the enzyme is severely but not completely inhibited. This inhibition remains constant to 1.7×10^{-4}M, after which the inhibition becomes even greater. Neither inhibition is reversed by ApA, but the first stage is completely abolished by BME. The second stage of inhibition is not reversed by BME, as we shall see from the $HgCl_2$ data. Higher concentrations of PMB could not be used because of solubility problems. The phosphorolytic activity (not shown) is reversibly inhibited by PMB at higher concentrations than the ADP activity, and is also irreversibly inhibited at the highest concentration shown. Further, the inhibition of the phosphorolytic activity is less extensive than that for the ADP activity.

I attempted to determine whether ApA or one of the substrates of the incorporation activity, i.e. ADP or $MgCl_2$ might protect the enzyme from PMB inactivation. The

procedure was to carry out the PMB treatment as above and to include, in addition, the reagent in question, at a concentration such that when the assay was performed, that reagent was at the correct concentration. Thus, for ApA the concentration during PMB treatment was 5 mg/ml, so that the assay contained the usual 100 µg, for ADP the incubation concentration was 200 mM (40 mM in assay), and for $MgCl_2$, 50 mM (10 mM in assay). The PMB concentrations in the figures are again those present during the one hour incubation.

A control experiment was carried out for comparison purposes (Fig. 26a). Results are as expected: inhibition by PMB, reversed by BME but not by ApA. Figure 26b shows the result when ApA was present along with the PMB. ApA does not protect the enzyme from PMB, and it is even possible that it enhances the inhibition slightly. The inhibition is still reversible by BME.

However, the presence of ADP quite definitely alters the action of PMB (Fig. 26c). ADP protects the enzyme from inactivation at $2x10^{-5}$M PMB, and seems to do so even at $2x10^{-4}$M PMB. The inhibition remains reversible. Not so with $MgCl_2$ (Fig. 26d). Not only does $MgCl_2$ greatly enhance the inhibition, but it also seems to make it more irreversible at $2x10^{-4}$M PMB. In neither of the above cases does ApA have any appreciable effect in reversing the inhibition. The results are clear: ApA has no affect on the PMB inhibition; ADP partially protects the enzyme from PMB; and

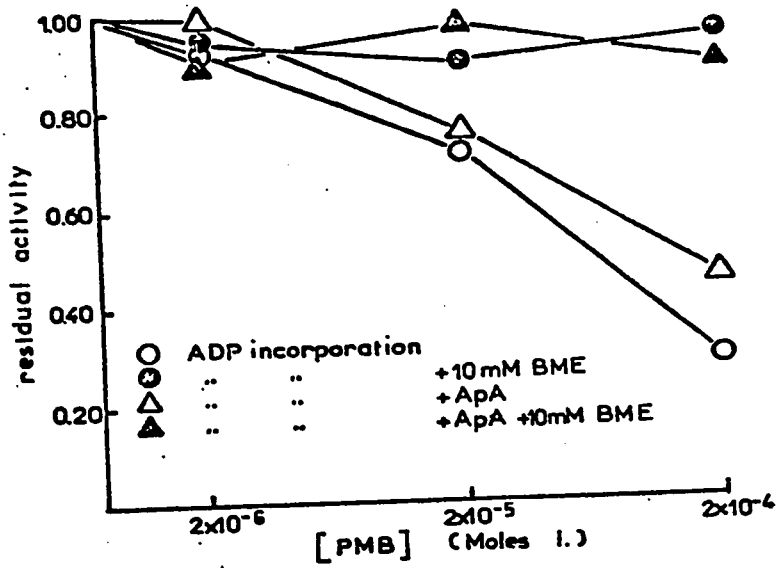


FIG. 26 (a)

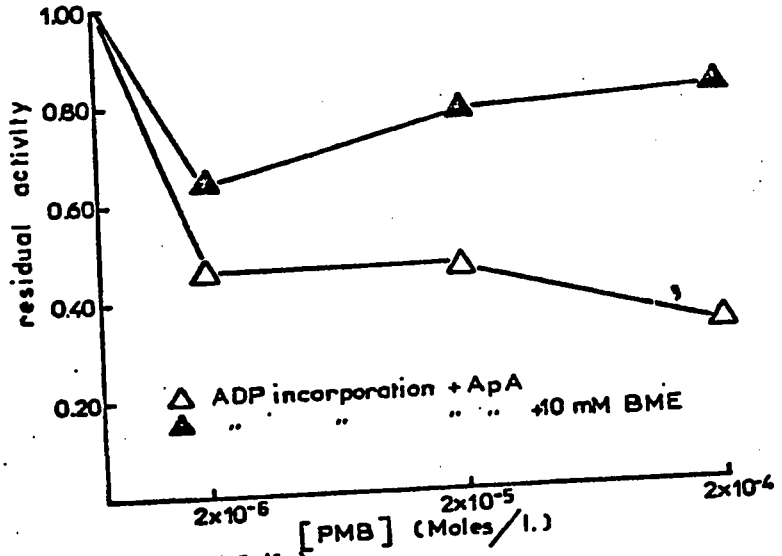


FIG. 26 (b)

FIG. 26. Combined effects of PMB with individual constituents of standard ADP-incorporation assay

a) PMB alone
b) PMB and ApA

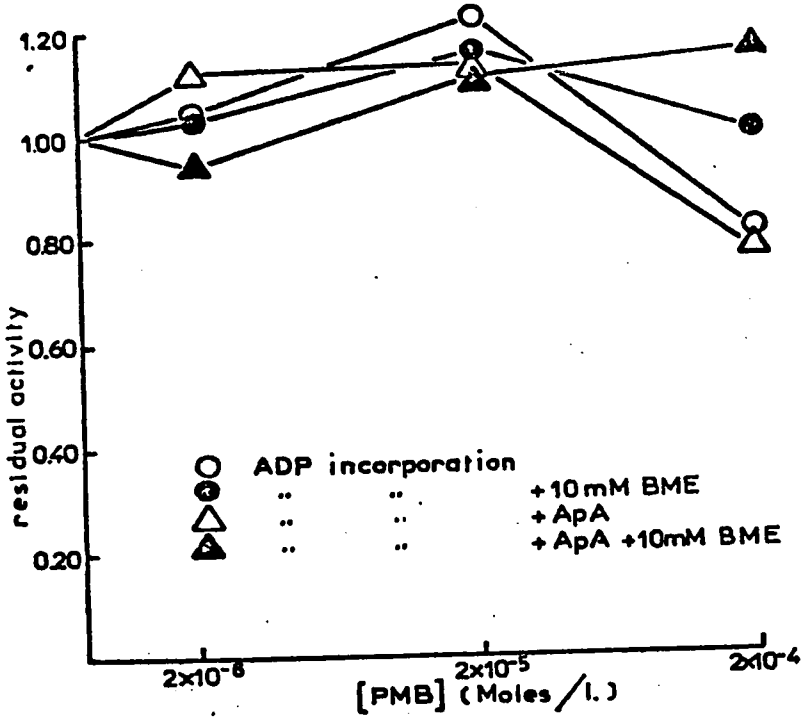


FIG. 26 (c)

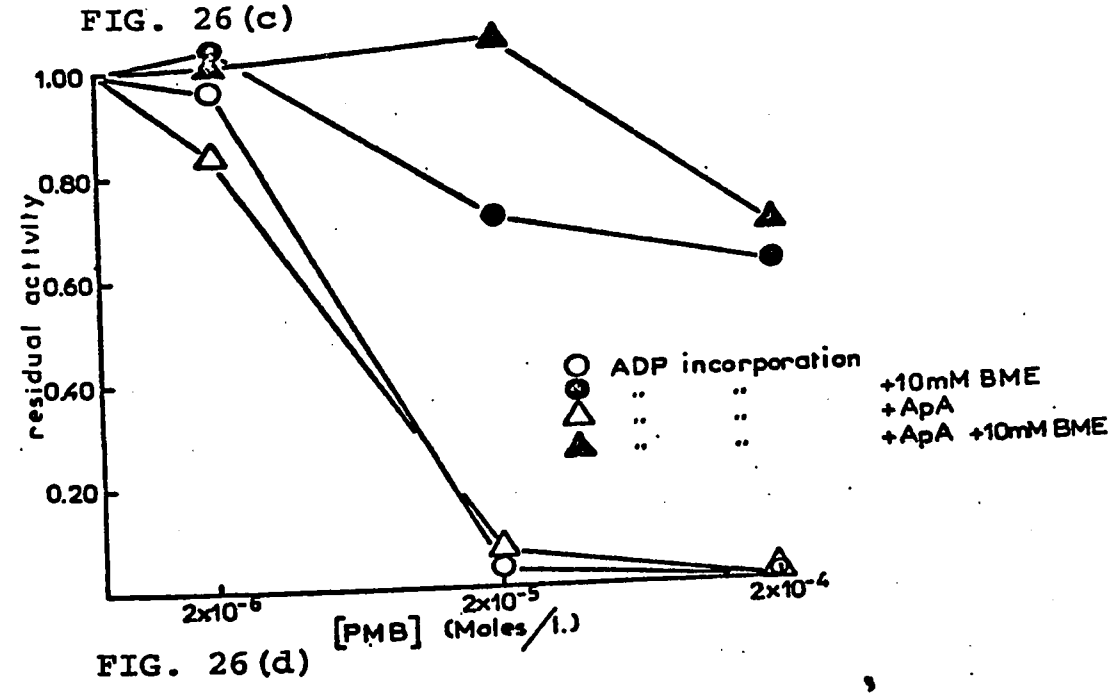


FIG. 26 (d)

FIG. 26 (cont'd). Combined effects of PMB and individual constituents of standard ADP-incorporation assay

c) PMB and ADP
d) PMB and $MgCl_2$

$MgCl_2$ enhances the inhibition by PMB. However, the possibility remains that ApA plays some indirect part. An aged, highly-purified enzyme with a large primer requirement was stimulated somewhat less by ApA after treatment with PMB than it had been before treatment, similar to the observations mentioned for oxidation by air or oxidizing reagents.

As with other reagents the inhibition is not solely due to inhibition of the assay by the PMB present. The pure enzyme was treated with 0.5 mM PMB for one hour, and samples were dialyzed against either TE 8.2 or TEB 8.2 buffer. Control samples of the enzyme not treated with PMB were similarly dialyzed (Table 32). As we can see from the figures for the ADP activity before PMB treatment and dialysis, the enzyme was appreciably stimulated by both BME and ApA. PMB treatment alone caused a large increase in the BME stimulation but not the ApA stimulation, as expected. Dialysis of the untreated enzyme against TE 8.2 buffer, oxidized the enzyme further, but this was completely prevented by dialyzing against TEB 8.2. The PMB-treated enzyme dialyzed against TE 8.2 buffer is even more oxidized, indicating that some part of the oxidation was due to PMB, rather than air. The oxidation is completely reversed by BME in the assay, or prevented by dialyzing against TEB 8.2 buffer. In no case does ApA appear to reverse the oxidation. All the different samples behaved identically on polyacrylamide gel electrophoresis, so it seems clear that PMB

TABLE 32

Effect of PMB-treatment and dialysis on pure PNPase

	Residual ADP-incorporation activity with addition to assay of:			
	No addition	10 mM BME	100 µg ApA	10 mM BME 100 µg ApA
PRE-DIALYSIS				
control	0.82	1.00	0.96	1.18
PMB-treatment	0.20	1.00	0.31	1.16
POST-DIALYSIS				
control TE 8.2	0.35	0.77	0.40	0.88
control TEB 8.2	1.03	0.94	1.18	1.14
PMB-treated TE 8.2	0.25	0.94	0.27	0.99
PMB-treated TEB 8.2	1.00	1.00	1.18	1.10

treatment causes no major irreversible changes on the structure of the enzyme.

v) Mercuric chloride

$HgCl_2$, because of its higher solubility, gave much more dramatic results than PMB (Fig. 27). This experiment was carried out in the same manner as that in Figure 25, substituting $HgCl_2$ for PMB. There seems to be no effect whatever up to $5x10^{-6}M$ $HgCl_2$; then there is a completely reversible inhibition. At $5x10^{-2}M$ $HgCl_2$, the enzyme has no activity, and the lost activity is only partially restored by BME. At this concentration there was heavy precipitation in the non-BME assay tubes and cloudiness in the BME assay tubes, so the effect is probably not strictly chemical. Again, the lost ADP-incorporation activity is restored by BME but not by ApA. The phosphorolytic activity is somewhat less sensitive to $HgCl_2$ at the lower concentrations than is the ADP-incorporation activity, but at the higher concentrations, the $HgCl_2$ effect is the same for both the activities. It is interesting to compare this experiment with that shown in Figure 25, in that the two resemble each other so closely.

The enzyme was treated with $HgCl_2$ in an experiment similar to that shown in Table 32 (Table 33). The pure primer-stimulated enzyme was treated with $5x10^{-2}M$ $HgCl_2$, then samples were dialyzed, either against TE 8.2 buffer,

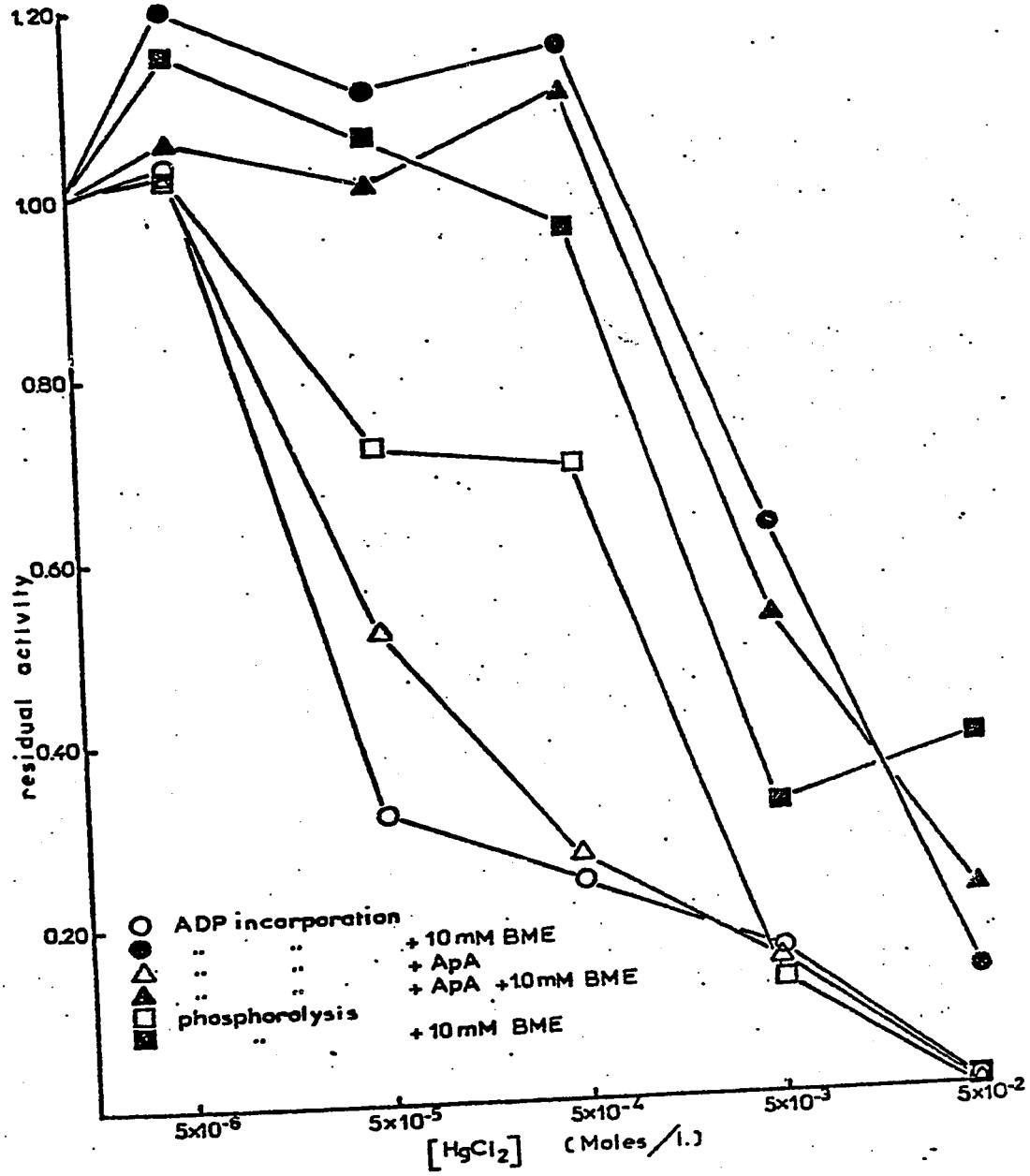


FIG. 27. Effect of $HgCl_2$ on pure PNPase

TABLE 33

Effect of $HgCl_2$-treatment and dialysis on pure PNPase I

	Residual ADP-incorporation activity with addition to assay of:				Residual phosphorolytic activity with addition to assay of:	
	No addition	10 mM BME	100 µg ApA	10 mM BME 100 µg ApA	No addition	10 mM BME
PRE-DIALYSIS						
control	1.11	1.00	1.21	1.29	0.89	1.00
$HgCl_2$-treated	0	0.024	0	0.020	-	-
POST-DIALYSIS						
control TE 8.2	0.45	0.72	0.48	0.77	0.38	0.58
control TEB 8.2	0.92	0.90	1.03	1.02	0.67	0.79
$HgCl_2$-treated TE 8.2	0	0	0	0	0	0.084
$HgCl_2$-treated TEB 8.2	0.13	0.10	0.12	0.13	0.12	0.16

or TEB 8.2 buffer. A control enzyme, not treated with $HgCl_2$, was similarly dialyzed. The dialysis alone caused appreciable oxidation which was completely reversed by BME. The $HgCl_2$ treatment completely abolished the ADP-incorporation activity, and even dialysis against BME restored only a small fraction of the activity. However, some phosphorolytic activity remained, but only in the presence of BME, so the enzyme was not completely denatured.

The experiment was repeated with a different pure enzyme preparation, which was not stimulated by BME or ApA (Table 34). In this case, the $HgCl_2$-treated enzyme was dialyzed against TE 8.2 buffer, then two samples were withdrawn and dialyzed--one against TE 8.2 buffer, the other against TEB 8.2 buffer. As we can see, the inactivation by $HgCl_2$ is less extensive, and the enzyme retains appreciable activity with BME. An interesting point is, that after standing a further two weeks, the $HgCl_2$-treated, TE 8.2-dialyzed enzyme had lost all its activity both with and without BME, whereas the $HgCl_2$-treated, TEB 8.2-dialyzed fraction retained 24% of its original activity, and was not stimulated by either BME or ApA.

The $HgCl_2$ treatment also caused irreversible changes in the physical properties of the enzyme. Polyacrylamide gel electrophoresis of the $HgCl_2$-treated TE 8.2-dialyzed enzyme revealed no activity, but staining for protein showed both the original enzyme band and a new faster-moving

TABLE 34

Effect of $HgCl_2$-treatment and dialysis on pure PNPase II

	Residual ADP-incorporation activity		residual phosphorolytic activity	
	No addition	10 mM BME	No addition	10 mM BME
PRE-DIALYSIS				
control	0.97	1.00	0.92	1.00
$HgCl_2$-treated	0.03	0.40	0	0.34
$HgCl_2$-treated TE 8.2	0	0.043	0.059	0.18
$HgCl_2$-treated TEB 8.2	0.16	0.23	0.40	0.56

band. The bands were very indistinct and between the two was a diffuse area of stain. Electrophoresis of the $HgCl_2$-treated TEB 8.2-dialyzed enzyme revealed the same protein pattern, but the activity gels had an indistinct band in the region of the original enzyme band. It is obvious that treatment with these high concentrations of $HgCl_2$ causes irreversible changes in both the physical and chemical properties of PNPase.

vi) Other reagents

Another sulfhydryl-specific reagent is 5,5'dithio-bis (2-nitrobenzoic acid) (DTNB, Ellman's reagent) (61). Like PMB, it binds sulfhydryl groups reversibly and forms the basis of an assay for these groups. In concentrations ranging to $5x10^{-3}M$, DTNB has no effect on the enzyme. At $5x10^{-2}M$, DTNB inhibits the ADP-incorporation activity 50% and the phosphorolytic activity 34%, in each case reversible by BME. Thus in its action on PNPase it is quite different from PMB or $HgCl_2$.

Yet another reagent, N-ethylmaleimide (NEM) (181) binds sulfhydryl groups irreversibly. It has no effect on PNPase at $5x10^{-5}M$, inhibits the ADP-incorporation activity 20% at $5x10^{-4}M$ and 30% at $5x10^{-2}M$. Thus there is almost constant inhibition after $5x10^{-4}M$ NEM. This inhibition is not reversed at all by BME. Curiously the phosphorolytic

activity is unaffected by NEM, even at $5x10^{-2}M$. Again the result is much different than that from PMB and $HgCl_2$.

DISCUSSION

My contributions to the understanding of polynucleotide phosphorylase can be summarized as follows. Firstly, I have found that the enzyme can undergo a reversible oxidation-reduction, and that the site of this phenomenon is a relatively reactive sulfhydryl group. Secondly, this discovery has permitted the development for the first time of a reproducible method for purifying the enzyme in high yield. Thirdly, studies of the highly-purified enzyme have led to certain insights into the physical structure of the pure A. vinelandii PNPase, and also of the modified (aged or trypsin-treated) enzyme. This, of course, is interconnected with studies on the catalytic properties, especially with regard to the primer-requirement. Finally, I have, for the first time, unequivocally demonstrated the existence of a transnucleotidation activity in a pure PNPase, which has important implications in terms of the mechanism of the reactions catalyzed by PNPase.

I believe that the maintenance of the enzyme in the reduced state at all times is primarily responsible for the improved reproducibility of my purification procedure, and the high yield and stability of the pure enzyme. Certainly, such factors as temperature, pH, and presence of EDTA do not seem to significantly affect various stages of the

purification. My preliminary work showed that the absence of BME in the buffers led to poor purifications, poor yields and unreproducible results. This confirms the results reported in the literature (166,214). It is clear to me that these authors had been attempting to purify a partially-oxidized enzyme, and therefore their data are suspect, at least in part. Certainly, published values for substrate requirements are in error, as I have shown, since the oxidized and reduced enzymes differ in this respect. I suspect that these difficulties in reproduction of results are the primary reason why A. vinelandii PNPase has not been studied in as much detail as the M. luteus and E. coli enzymes.

A very important factor, in my opinion, is the reproducible high quality of the various Sephadex derivatives. Many published purification procedures involve the notoriously-irreproducible calcium-phosphate gel fractionation, or DEAE-cellulose columns. My preliminary work certainly confirmed this, as have other reports (111). Careful control of the conditions of certain steps in the purification conditions I have described (e.g. column size, flow-rate, preparation of columns, gradients, etc.) is critical, if severe mechanical problems and consequent poor yields are to be avoided. The exact method described does not appear to be generally applicable to purification of PNPase from other sources, since, for instance, the enzyme

of E. coli would not bind to the first DEAE-Sephadex column in the conditions described.

My results for the pH-optimum and effect of salt confirm those previously found for PNPases of the A. vinelandii-E. coli type. However, in the polymerization reaction the activity depends on the ADP/Mg^{++} ratio, rather than on each of the two taken individually, and in this case, the published figures are in error.

In a review article by Grunberg-Manago (83) there is a figure which shows that the optimum ADP/Mg^{++} ratio is approximately 2 for E. coli PNPase, approximately 3 for A. vinelandii PNPase, and approximately 4 for M. luteus PNPase. A later publication (237) reports the ratio for A. vinelandii PNPase to be 1-2. My own finding for the pure, reduced A. vinelandii PNPase is that the ratio is 4, similar to that for M. luteus PNPase. Further, I found that the ratio for the oxidized enzyme is 2. This strongly suggests that previous authors may have been dealing with a partially-oxidized PNPase, and that the variation in the published results may be due to use of varying mixtures of the oxidized and reduced forms of the A. vinelandii enzyme. A detailed re-examination of the kinetic data published for A. vinelandii PNPase will be needed to clarify this point further, as it seems certain that all published studies of the kinetics of nucleoside diphosphate polymerization, and, to a lesser extent, phosphorolysis catalyzed by the

A. vinelandii must be considered suspect. A. vinelandii PNPase prepared by my method is an ideal candidate for the general studies of the kinetics and mechanisms especially of the de novo polymerization, since it is much more stable in every way than PNPase from other sources, especially with respect to development of primer requirement.

I did, in fact, make some preliminary attempts to study the kinetic behaviour of A. vinelandii PNPase. The crucial information needed for this sort of study is the concentration of "free" ADP and "free" Mg^{++} in the reaction mixture, that is, the amounts not involved in an ADP-Mg^{++} complex. The method quoted for measuring "free" Mg^{++} involves the use of 8-hydroxyquinoline (151), but I found that the procedure was accurate only for Mg^{++} concentrations an order of magnitude less than those found in the standard assay. Reference 237 refers further to results obtained by Dr. P. Dauta, published in a thesis presented at the University of Paris, and unavailable in the general scientific literature. It is possible that he developed a modification which allowed the determination of the various kinetic constants. I personally did not expand on my preliminary kinetic data, since this in itself would be a major separate study. However, I must point out that any valid kinetic experiments must await the publication or development of a procedure for determining the free Mg^{++} concentration in the conditions of assay, with unequivocal

demonstration that the process of measuring this value does not alter the equilibrium between free and bound Mg^{++}.

Physically, the A. vinelandii enzyme differs in some respects from other PNPases. The most obvious difference is the presence of a nucleic acid "contaminant", which has been noted before (166,214). It now seems clear that it is not a contaminant, but perhaps a covalently-bound prosthetic group, since it cannot be removed without also destroying the activity of the enzyme. This is quite different from E. coli or M. luteus PNPase, and is of unknown significance. I must point out that in published purifications of E. coli PNPase, the ratio A_{280}/A_{260} on which the claim that nucleic acids are absent is based, is very high in early stages of the preparation. Further, in my own preliminary experiments with E. coli PNPase, I obtained ratios which were so high as to be completely beyond the range of the Warburg and Christian technique. While such a high O.D. ratio may indicate that relatively low amounts of typical nucleic acids are present, it is also possible that some unusual nucleic acid or other U.V.-absorbing contaminant may be present. For instance, polynucleotides with an abnormally high cytidine content would be expected to give high absorbancy readings at 280 nm, since cytidine absorbs strongly in this region. Further, prior to my work no one has ever directly measured the nucleic acid content of any PNPase, for instance by the orcinol procedure. Thus the

claims that PNPase is free from nucleic acid may be in error. The "anomalous" band is also puzzling. It must be bound to the PNPase in some way, since it accompanies the enzyme through the various purification steps. However, the binding is rather loose and not covalent, since it can be separated from the enzyme by electrophoresis. It is certainly not essential to the activity of PNPase, although it is possible that it may be an activator, if only because the yield of enzyme units is so poor after electrophoresis. Its existence has not been noted in any other PNPase preparation, probably because all other authors use Amido Black rather than Coomassie Blue, for staining protein. It is resistant to a broad range of degradative enzymes, so its nature remains in doubt, but a recent report (35) has shown that phospholipids can be stained by Coomassie Blue, and that this anomalous staining is enhanced by such fixatives as trichloroacetic acid. It therefore seems possible that the "anomalous" component is in fact a membrane fraction. This is particularly interesting in view of the fact that it has been speculated that PNPase is membrane-bound *in vivo* (1, 190).

A very interesting result is that obtained from SDS-gel electrophoresis. My data suggest that *A. vinelandii* PNPase possesses only a single type of subunit, of M.W. around 7×10^4 daltons. This is very encouraging in light of the results quoted by Thang (215), that *E. coli* PNPase also

had a 6.5×10^4 dalton subunit. However, he also claimed to have observed a 3×10^4 and a 1×10^5 dalton subunit, neither of which I can detect. Thang's results have been seriously questioned in a recent report by Lehrach et al (137). These authors could detect neither the 3×10^4 nor the 6.5×10^4 dalton species, but only the 9.5×10^4 dalton species. On close reading of their report, it is clear that they base their estimations of molecular weight with reference to the σ-subunit of E. coli RNA polymerase which they give as 9.5×10^4 daltons (note the misprint in Figure 3 of their report). Since the molecular weight of this protein has been reported variously as $8.5\text{-}9.5 \times 10^4$ daltons (28), and since SDS gels have poorer resolution than normal gels, it is possible that the M.W. of my "light" species is somewhat higher than the one I have given and thus in the range reported for the E. coli PNPase subunit. With further refinements of the SDS-gel technique, which have occurred since my work was completed, it may be possible to resolve this problem.

The focal point of my work, of course, is the discovery of the reversible oxidation-reduction. I found that the partially-oxidized enzyme was very difficult to purify, since it was eluted from ion-exchange columns in very broad peaks. This could explain the difficulties reported by other authors in purifying A. vinelandii PNPase. It would also explain the low yields of units reported, as

it seems likely that they were measuring the activity of an oxidized enzyme.

Cl. perfringens PNPase is believed by some authors to undergo a reversible oxidation-reduction, but this involves a monomer-dimer transition (96). Such is not the case with A. vinelandii PNPase, where the oxidation is intramolecular. The air-oxidized enzyme does not differ physically in any way from the native, reduced form. In fact, the only obvious difference is that the rate of the incorporation assay and the ADP-Mg^{++} requirement are both much lower for the oxidized enzyme. Treatment with such oxidizing agents as GSSG, potassium ferricyanide and p-benzoquinone mimics the air-oxidation insofar as the effect on the catalytic properties is concerned, but it is probable that they also modify other functional groups on the enzyme so that the physical properties are drastically changed.

The results with sulfhydryl-specific reagents are much more conducive to speculation. It is clear from the PMB and $HgCl_2$ results that there are at least two different classes of sulfhydryl groups on the enzyme. The first type consists of groups which are quite sensitive to sulfhydryl-specific reagents, and is involved in some way with the catalytic function of the enzyme. This is not to say that this class necessarily forms a part of the active site, but simply that alteration of these groups affects the activity, possibly through a conformational change. This class also

includes those groups which I believe to be the ones involved in the reversible oxidation. There is some indication that these groups may be partially shielded or relatively inaccessible, despite their reactivity, because such large substituting reagents as DTNB and NEM do not react as readily as the Hg moiety of PMB and $HgCl_2$. In general, alterations to these groups are reversible. The second class of sulfhydryl groups is substituted by sulfhydryl-specific reagents only at relatively high concentrations, i.e. these groups are less reactive. These groups are also less intimately involved with the activity of the enzyme. Further, at the concentrations of reagents required to react with these groups, the enzyme is irreversibly inactivated. My results do not permit an estimate of the relative abundance of these two classes of groups nor of their localization.

I must stress that changes in either class of sulfhydryl groups are completely independent of primer requirements, contrary to conclusions drawn in a recent review (129), misquoting my published results (76). A. vinelandii PNPase is quite unlike M. luteus PNPase, where oxidation or substitution at a certain sulfhydryl causes an increase in primer-requirement (132). It seems that for M. luteus PNPase, sulfhydryl groups are in some way involved in primer-binding or chain-initiation. With A. vinelandii PNPase, my data clearly show that sulfhydryl

groups are involved only in the chain-elongation reaction, since primers do not reverse the effect of "oxidation." However, preliminary data suggest that the primer-requirement may be lower in the oxidized enzyme. It is also possible that the presence of ApA potentiates the reaction with PMB. It therefore seems that although sulfhydryl groups are not directly involved in primer-requirement, there is a very indirect relationship between the two. It is probable that this inter-relationship is caused by conformational changes rather than direct modification of binding or active sites, and may be merely a reflection of the reduced catalytic efficiency of the oxidized enzyme.

On the other hand, the sulfhydryl groups are intimately involved with the catalytic site of the enzyme. ADP protects the enzyme from PMB while $MgCl_2$ potentiates the inactivation by PMB. Since PNPase binds ADP and Mg^{++} in two separate steps (237), it is likely that the binding of each of these involves a different conformation change in the enzyme. Since free sulfhydryl groups are essential to the activity, probably for the maintenance of an active conformation, it is possible that ADP restricts the accessibility of these groups, while Mg^{++} has the opposite effect. It is not possible, of course, to measure the effect of both reagents taken together, since in this case polymerization takes place, introducing an extraneous factor. It would be interesting to study the inter-relationships of

PMB, Mg^{++}, and some ADP-analogue which would be bound but not polymerized.

In the case of M. luteus PNPase, the effect of trypsin is connected with the sulfhydryl groups, since it is only after trypsin-treatment that the reversible oxidation is evident. I use the term "oxidation" in a very broad sense, since the M. luteus PNPase has never been directly shown to undergo a reversible air-oxidation, but rather a reversible substitution by sulfhydryl-specific reagents. It is possible that the native M. luteus PNPase (2.6×10^5 daltons) is degraded by trypsin in a way such that certain free sulfhydryl groups tend to form disulfide linkages (128), and the enzyme is inactivated. The "active conformation" of the enzyme "core" (2.2×10^5 daltons) is then restored by regenerating the original free sulfhydryl groups. With A. vinelandii PNPase the situation is quite different. The native PNPase (2×10^5 daltons) requires free sulfhydryl groups for activity, but trypsin-treatment causes an effect independent of these groups. It is interesting to speculate whether the native A. vinelandii PNPase may not be similar to the "trypsinized" M. luteus enzyme, and whether the latter might not resemble the "trypsinized" A. vinelandii PNPase, if it were more extensively degraded. This could also account for the observation that pyrimidine diphosphate polymerization is preferentially affected by limited trypsin-treatment of M. luteus PNPase ("differential effect")

unlike *A. vinelandii* PNPase.

An important point is that nucleic acids have been found to protect the *M. luteus* enzyme from degradation by trypsin (65), although it is not clear whether there is any connection with the differential effect. However, the differential effect is reported only for relatively-impure *M. luteus* PNPase preparations (65), and has not been repeated for the pure enzyme (160). Since impure enzyme preparations obviously have higher proportions of nucleic acids than pure ones, a protective effect can be expected. This would explain why the differential effect was observed after trypsin-treatment of impure PNPase preparations, but not of pure *A. vinelandii* PNPase.

Thus, to summarize the conclusions drawn from the oxidation and proteolysis of PNPase, it appears that *M. luteus* is degraded by trypsin in a way such that approximately 400 amino acid residues (4×10^4 daltons) are removed. All the functions involved in the catalytic process are affected by this degradation, but the chain-initiation activity is shown to depend on the maintenance of certain free sulfhydryl groups, rather than directly on the portion of the protein-chain which has been removed. In *A. vinelandii* PNPase, it is the chain-elongation activity which is dependent on free sulfhydryl groups, and the chain-initiation activity is independent of the sulfyhydryl groups.

I have observed qualitatively similar results,

caused simply by storage of the pure A. vinelandii PNPase. The pure PNPase develops a primer requirement with age, accompanied by changes in the physical properties. The latter are very small and perhaps only a change in conformation rather than a loss of a small portion of the polypeptide chain. Similar changes have been reported for E. coli PNPase (137), and this may be due to an endogenous protease (216). With A. vinelandii PNPase I have been unable to detect any protein band other than the enzyme, so I do not believe that such a protease is present. It is interesting that the changes in primer-requirement I observe are much smaller and slower than those reported for E. coli and M. luteus PNPases. It seems probable that my enzyme is inherently more stable than these latter two.

My results for the CDP-polymerization, phosphorolysis, and exchange activities, present special problems in interpretation. I cannot make the blanket statement that the pyrimidine diphosphate-polymerization is in all cases more sensitive to modifications in the enzyme, since this is not the case for trypsin-treatment. Nevertheless, aging and oxidation do preferentially affect this activity. It is possible that, aside from the considerations mentioned earlier, trypsin-treatment is an extreme case of aging, and that the "differential effect" observed for M. luteus PNPase, and in early work with A. vinelandii PNPase, is caused by very mild trypsin-treatment. This, however, does not

explain the differential effect of oxidation, unless one assumes that pyrimidine diphosphates present special problems as substrates for the enzyme, and require a very narrowly-specified active conformation for activity.

The phosphorolytic activity is less affected by modifications to the enzyme than are the other activities. Because phosphorolysis is considered to be the metabolically-significant activity *in vivo*, it is not unreasonable to assume that the enzyme is inherently capable of catalyzing this reaction, but requires special conditions for the other reactions (compare the PNPase isolated from animals, reference 68). Thus, I speculate that the more "reduced" the enzyme, that is, the more "open" the configuration of the enzyme protein, the higher the phosphorolytic activity. This would account for the stimulation of the phosphorolytic activity by BME at concentrations which inhibit the other activities. Nevertheless, trypsin-treatment does reduce the phosphorolytic activity of the enzyme. It has been shown for *E. coli* PNPase (216) that partial degradation of the enzyme protein results in a higher K_m for polyA in the phosphorolysis reaction. This would also account for the results observed for *A. vinelandii* PNPase.

A very curious observation is that the exchange activity of the pure *A. vinelandii* PNPase is not stimulated by oligonucleotide primers, but rather is inhibited. Although early work with various PNPases showed that

oligonucleotides were not necessary for exchange (121), more recent reports suggest that a primer is absolutely necessary, whether exogenous, or synthesized in situ (45). It is possible that for pure native A. vinelandii PNPase, de novo polymerization is the preferred reaction rather than exogenously-primed synthesis, and that exogenous primers inhibit the de novo synthesis of a primer for exchange. I must admit that I have no experimental evidence to support this hypothesis, and it certainly does not agree with the conclusions drawn for other PNPases. One saving factor is that, to my knowledge, no one has ever shown conclusively that in unprimed exchange any endogenous primer is synthesized. In fact, it would be very hard to demonstrate the formation of endogenous primer, since it need be present only in catalytic amounts for the exchange reaction. Alternatively, one can propose that A. vinelandii PNPase acts by a different reaction mechanism than that proposed for other PNPases.

Such is the case when one considers the transnucleotidation activity. As I pointed out in the "Introduction" section, the generally-held scheme for the reaction mechanism of PNPase cannot apply to the transnucleotidation reaction, because this reaction cannot proceed through an enzyme-NDP intermediate. No NDP can be formed in the reaction, simply because there is no phosphate present in the reaction medium. In addition, this activity cannot involve any

de novo synthetic step.

It is possible that PNPase is a phosphorylated enzyme, providing the necessary catalytic amounts of phosphate, but the evidence for this is tenuous at best (216). In this postulated scheme for transnucleotidation, the phosphorylated enzyme would bind two molecules of oligonucleotide, the terminal residue of one would be cleaved with the addition of the "endogenous" phosphate, and this residue would immediately be added to the other oligonucleotide, with retention of the "endogenous" phosphate on the enzyme. The "endogenous" phosphate would probably not have to be released from its binding site at any time and thus would not be released into the medium. A corrollary of this scheme is that the binding sites for the two oligonucleotides would have to be spatially very close, and near to the active catalytic site of the enzyme. Another obvious requirement is that phosphate would have to be much more strongly bound than oligonucleotide in the correct conditions for transnucleotidation. Even in phosphorolytic conditions this does not appear to be the case as I have discussed in the "Introduction," since the K_m for phosphate is much higher than that for polynucleotides, although this does not necessarily apply to any "endogenous" phosphate. Further, I carried out my transnucleotidation experiments in the conditions normally used for NDP-incorporation assays, in which one would expect phosphate to be even more weakly

bound.

I personally favour an alternative explanation, that the reactions catalyzed by PNPase proceed through an enzyme-NMP intermediate. One attraction of this scheme is that it is conceptually easy to visualize, and is the simplest one that accounts for all the activities attributed to PNPase. To recapitulate this scheme, in de novo polymerization an NDP molecule would bind to the enzyme as NMP with release of phosphate. This cleavage of phosphate might be the irreversible de novo polymerization step, and the NMP must also not be released from the enzyme. This would also account for the observation that there is a 5' monophosphate on the product of PNPase-catalyzed polymerization (100). Next, a second NDP would bind at a separate site in the same way, and would be attached to the first NMP to give the initial "primer." It is more probable that this condensation is the irreversible step in de novo synthesis, rather than the initial binding. After this point the elongation continues with sequential addition of "activated" NMP residues to the pre-formed exogenous or endogenous primer. The unprimed exchange reaction would then be the binding of NDP as NMP, followed by displacement by exogenous inorganic phosphate. The transnucleotidation reaction would involve the "activation" (or cleavage) of the 3' terminal NMP residue of one oligonucleotide, followed by its addition to the 3' terminal end of another oligonucleotide. Two

possibilities exist for this latter step; either the oligonucleotide to be elongated is attached at some other site on the enzyme, possibly a binding site for oligonucleotide in phosphorolysis, or the second oligonucleotide is bound at the site vacated by the cleaved oligonucleotide. I favour the first possibility, since it has been suggested that PNPase does possess multiple binding sites for oligonucleotides (90,160) and it has been shown that, in general, the rates of catalysis by PNPase are much faster than the rate of binding of oligo- and polynucleotides (90). Finally, the mechanism for phosphorolysis would be similar to that for exchange, in that the "activated" 3' terminal residue would be displaced by inorganic phosphate. One can speculate that, in fact, two exchange activities are present: a "true" exchange involving an enzyme-NMP complex, which might be present in fresh, fully-active enzyme preparations and an "apparent" exchange activity which would involve a reversible polymerization-phosphorolysis. The "optimum" for each of these exchanges might be different, and the "optimum" reported in the literature might in fact favour only one of the activities.

This scheme is not generally accepted by other authors and, in fact, has been vigorously denied. There are three basic arguments involved. First, Hendley and Beers (103) have prepared various schemes for the reaction mechanism, involving enzyme-AMP intermediates. The kinetic

equations for phosphorolysis then suggest that "polyA competes with P_i for the enzyme-AMP complex" i.e. that exchange and phosphorolysis are competitive reactions and that polyA inhibits exchange. This effect has not been observed. Secondly, the existence of an enzyme-NMP complex cannot be shown by equilibrium-dialysis experiments (20). Thirdly, dADP exchange occurs only in the presence of a primer (45), although other authors deny this (121). It appears, however, that radioactivity from labelled dADP is strongly bound to PNPase, but appears to be polymeric in nature, in widely varying conditions of salt, Mg^{++}, pH, temperature and time of incubation (45).

There is at present no conclusive way to refute these results. Although A. vinelandii PNPase may be quite different from M. luteus PNPase, it seems quite similar to the E. coli enzyme, yet the latter two are assumed to be the same with respect to reaction mechanism. I could speculate that the transnucleotidation activity has a different mechanism than the other activities, but I feel that this is a trifle premature. It is quite clear that kinetic studies of the sort performed with E. coli and M. luteus PNPases must be carried out with the pure A. vinelandii PNPase, in a carefully-controlled oxidation state, and that the transnucleotidation must be demonstrated in the former two, before a final decision can be reached.

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